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(54) Title: CYTOCHALASIN COMPOSITIONS AND THERAPEUTIC METHODS

(57) Abstract

The present invention is directed to naturally occurring and synthetic cytochalasin compositions and therapeutic treatments utilizing these compositions. More specifically, the present invention relates to certain synthetic analogues of Cytochalasin B (CB) and sustained release formulations containing a cyctochalasin, for example, cytochalasin B and other natural cytochalasins, for example, Cytochalasin D, E, H or J, among others, and one or more of its synthetic analogues. This invention also relates to the surprising discovery that the administration of cytochalasins including CB produces transient immunosuppression which is controllable by dose or route of administration and is reversible spontaneously or with the use of IL-2. Thus, a therapeutic regimen of cytochalasins may be used to treat the undesirable hyperimmunity of transplant patients and patients with autoimmune disease. In addition, anti-tumor therapy utilizing CB and other cytochalasins, and optionally antineoplastic agents other than cytochalasins may be significantly enhanced by combining the administration of these agents with effective amounts of IL-2 or other lymphokines for reversing the immunosuppression produced during administration of cytochalasins with other antineoplastic agents. This invention also relates to sustained release formulations utilizing liposomes or microcapsules which are effective for delivering high concentrations of cytochalasins and optionally, additional antineoplastic agents to the active site of the tumor without producing undesirable immunosuppression.

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CYTOCHALASIN COMPOSITIONS AND THERAPEUTIC METHODS

Field of the Invention

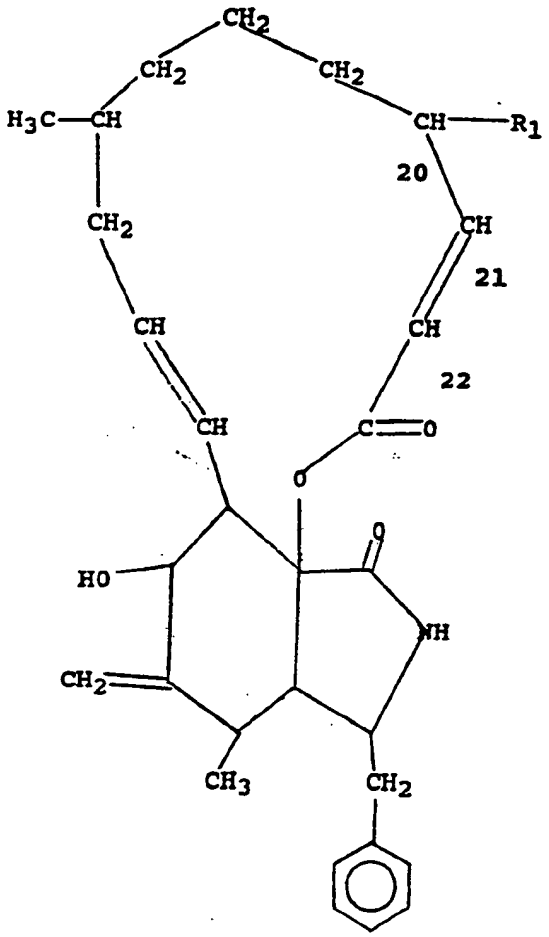
The present invention is directed to naturally occurring and synthetic cytochalasin compositions and therapeutic treatments utilizing these compositions. More specifically, the present invention relates to certain synthetic analogues of Cytochalasin B (CB) and sustained release formulations containing a cyctochalasin, for example, cytochalasin B and other natural cytochalasins, for example, Cytochalasin D, E, H or J, among others, and one or more of its synthetic analogues. This invention also relates to the surprising discovery that the administration of cytochalasins including CB produces transient immunosuppression which is controllable by dose or route of administration and is reversible spontaneously or with the use of IL-2. Thus, a therapeutic regimen of cytochalasins may be used to treat the undesirable hyperimmunity of transplant patients and patients with autoimmune disease.

In addition, anti-tumor therapy utilizing CB and other cytochalasins, and optionally antineoplastic agents other than cytochalasins may be significantly enhanced by combining the administration of these agents with effective amounts of IL-2 or other lymphokines for reversing the immunosuppression produced during administration of cytochalasins or cytochalasins with other antineoplastic agents.

This invention also relates to sustained release formulations utilizing liposomes or microcapsules which are effective for delivering high concentrations of cytochalasins and optionally, additional antineoplastic agents to the active site of the tumor without producing undesirable immunosuppression.

Background of the Invention

The cytochalasins, membrane- and transport-acting compounds (also having cytoskeletal-acting effects), include the congeneric cytochalasins A-M as well as the semi-synthetic derivatives 7,20-di-O-acetyl-cytochalasin B, 7-mono-O-acetyl-cytochalasin B, 21,22-dihydro-cytochalasin B and 21,22-dihydro-cytochalasin A, together with the related chaetoglobosins, constitute a class of more than 24 structurally and functionally related alkaloid metabolites produced by molds (Yahara et al., 1982, J. Cell Biol., 92:69-78, and Rampal et al., 1980, Biochem., 19:679-683). These substances are known to alter a wide variety of cellular functions in many different types of normal and neoplastic cells and tissues in culture (Miranda et al., 1974, J. Cell Biol., 61:481-500) and to exhibit differential effects in some cases between normal and neoplastic cell types (for references, see Lipski et al., 1987, Anal. Biochem., 161:332-340). The various congeners of these agents alter the biochemistry of fundamental cellular processes controlled by cytoskeletal and plasma membrane interactions. Some of the cytochalasins, for example, cytochalasin B (CB) pictured below, the most extensively studied of the congeners, inhibit hexose and amino acid transport in normal and neoplastic cells (Kletzien et al., 1973, J. Biol.
Chem., 248:711-719, and Greene et al., 1976, Exp. Cell. Res., 103:109-117, respectively).



The cytochalasins are also known to alter microfilament morphology (Schliwa, 1982, J. Cell Biol., 92:79-91), thereby affecting the cellular functions that depend upon microfilament biochemistry. Some of the cellular processes sensitive to the cytochalasins are phagocytosis, pinocytosis, cytokinesis, secretion, and exocytosis, as well as functions requiring movement and/or intercellular adherence including intracellular organelle movement and intercellular transport, cell motility, transport across tissue barriers, and a variety of immunological responses. The cytochalasins have only recently been shown to possess substantial activity against tumor growth and metastasis. Earlier studies on the in vitro effects of the cytochalasins on transformed (neoplastic) cells showed certain efficacy. For example,

O'Neill (1975, Cancer Res., 35:3111-3115), Medina et al. (1980, Cancer Res., 40:329-333), Kelly et al., (1973, Nature New Biol., 242:217-219), and Defendi et al., 1972, Nature New Biol., 242:24-26) showed differential effects between normal and neoplastic cells, involving nuclear division and cytokinesis resulting in multinucleation of neoplastic cells. However, the early in vivo chemotherapeutic studies did not reveal anti-tumor activity against intraperitoneal Ascites tumors (Katagiri et al., 1971, J. Antibiotics, 24:722-723, and Minato et al., 1973, Chem. Pharm. Bull., 21:2268-2277). In fact, intravenous administration of B16F10 cells, treated with CB in vitro showed an increase in extra-pulmonary metastases (Hart et al., 1980, J. Natl. Cancer Inst., 64:891-900).

In spite of the dramatic effects demonstrated by the cytochalasins and related substances on cells and tissues in vitro, few studies were conducted with these substances in vivo until only recently because of the lack of availability of sufficient quantities of these compounds, especially CB. Recent advances in methodologies for producing cytochalasins (see PCT Application US88/02095) have resulted in increased availability of larger quantities of the cytochalasins and in vivo administration and biostudies have become more routine.

Certain of these studies have shown that the cytochalasins, and especially, CB inhibit the growth and metastases of tumors, extend the tumor latency period, and extend host survival in a murine carcinoma and murine melanoma model system (Bousquet, et al., submitted for publication). These results lead to the proposal that Cytochalasins may function as chemotherapeutic amplifiers of the activity of known anti-tumor agents by virtue of the effects on cytoskeletal and plasma membrane functions. In recent studies, cytochalasins have been shown to affect leukocyte-mediated functions in vitro, the differentiation of cytolytic T lymphocytes and also to affect cell motility, cell adherence, phagocytosis and secretion (Wessels, et al. Science, 171, 135, 1971), factors which may affect host immune responses in vivo. Other studies have traced the

pharmacokinetics of CB in various tissues, including the spleen and liver, of mice injected with CB intraperitoneally (Lipski, et al., 1987, Analytical Biochemistry 161, 332-340). No study, however, has discussed or detailed the transient immunosuppressive effects of CB in vivo.

In recent years organ transplants and foreign tissue grafts have become commonly used for treating degenerative diseases or otherwise saving the lives of patients. However, the introduction of foreign tissue or a foreign organ into a patient is far from a risk-free procedure. Often, the tissue or organ is rejected by the patient's immune system, which detects the transplanted organ or grafted tissue as foreign. Currently-used immunosuppressive agents, for example, cyclosporin, are effective in reducing the chance of rejection by the patient, but often at the cost of reducing the patient's overall ability to ward off infection. In most cases, mortality from rejection of the transplant is not as high as the mortality rate from infection. The transplant patient can often die of infection usually or otherwise from non-athogenic diseases. In addition, there is often a high incidence of leukaemia or other malignancy with currently used immunosuppressive agents.

Certain of these currently used agents also find limited use in treating autoimmune diseases. The therapy suffers from the same limitations which occur when immunosuppressive agents are used to treat transplant or tissue graft rejection. The search, therefore, continues for more effective immunosuppressive agents that are effective at controlling a heightened immune response (hyperimmunity) in patients with transplanted organs, grafted tissue or an autoimmune disease without affecting the ability of the patient's system to fight off infection. The present invention, therfore, also relates to the surprising discovery that the cytochalasins, especially CB, exhibit significant transient, readily reversible immunosuppression in vivo.

Cytochalalasins, and especially CB, may become drugs of choice, either alone or in combination with known antineoplastic agents for

the treatment of tumors. CB, however, after administration, metabolizes quickly to the more highly toxic cytochalasin A (CA), as well as other metabolites. Past studies have suggested that CA is highly reactive with thiol groups, a fact which might explain the high toxicity associated with its use. It is believed that the metabolic oxidation of the 20-hydroxyl group of CB to the 20 keto group in CA to form the highly reactive enedione system, a chemical system which is thought to be highly reactive with thiols in enzymes and other proteins, may be primarily responsible for the high toxicity associated with the administration of CA. There is therefore a need in the art for an agent or group of agents which can take advantage of the pharmacological effects of the cytochalasins to inhibit the growth and metastasis of tumor cells, yet which decrease the general toxicity associated with the metabolism to the enedione system of CA.

The cytochalasins exhibit a number of pharmacological effects, many of which may be controlled by the amount and route of administration. These pharmacological effects may also be controlled by formulations utilizing liposome and microencapsulation techniques, or alternatively, by the administration of cytochalasins with, for example, lymphokines.

Liposomes are completely closed lipid bilayer membranes which contain entrapped aqueous volume. Liposomes are vesicles which may be unilamellar (single membrane) or multilammelar (onion-like structures characterized by multiple membrane bilayers, each separated from the next by an aqueous layer). The bilayer is composed of two lipid monolayers having a hydrophobic "tail" region and a hydrophilic "head" region. In the membrane bilayer, the hydrophobic (nonpolar) "tails" of the lipid monolayers orient toward the center of the bilayer, whereas the hydrophilic (polar) "heads" orient toward the aqueous phase. The basic structure of liposomes may be made by a variety of techniques known in the art.

The invention of the present application also relates to the discovery that the use of interleukin-2 (IL-2) in combination with the cytochalasins and optionally, other antineoplastic agents, eliminates the immunosuppressive effects of the cytochalasins. Interleukin-2, a lymphokine which is produced by normal peripheral blood lymphocytes and induces proliferation of antigen or mitogen stimulated T cells after exposure to plant lectins, antigens or other stimuli, was first described by Morgan, D. A., et al.., Science, 193, 1007 (1976). Il-2, in addition to its ability to induce proliferation of stimulated T lymphocytes, also modulates a number of functions of immunocytes in vivo. IL-2 is one of several lymphocyte-produced messenger regulatory molecules which mediate immunocyte interactions and functions.

Objects of the Present Invention

It is an object of the present invention to provide a therapeutic method for producing transient immunosuppression beneficial for treating disease states or conditions associated with heightened immune function, for example, autoimmune diseases or heightened immune response after an organ transplant or tissue graft. It is a further object of the present invention to provide antineoplastic formulations and therapeutic methods which employ cytochalasins in combination with IL-2 and optionally, with additional antineoplastic agents.

It is an additional object of the present invention to provide formulations employing liposomes or microcapsules and therapeutic methods utilizing these formulations for treating neoplasia. It is still a further object of the present invention to provide synthetic analogs of CB and therapeutic methods employing these analogs as anti-neoplastic agents and immunosuppressive agents. These and other objects and advantages of the present invention will become apparent from a review of the detailed description of the present invention.

Brief Description of the Figures

Figure 1 shows the maximum tolerated dosage (MTD) for CB administered to B6D2F1, CD2F1 or C57B1/6 via intraperitoneal (IP), sub-cutaneous (SC) and intravenous (IV) routes of administration using different vehicles. This information is adapted from Table 2 of U.S. Serial No. 208,199, filed June 17, 1988.

Figure 2 shows the effect of varying doses of CB administered to mice 19 hours before splenectomy on immunosuppression.

Figure 3 shows the effect of varying doses of CB administered to mice 3 hours before splenectomy on immunosuppression.

Figure 4 shows the effect of the IP administration of 50 mg/kg on the allogeneic rejection response in vivo.

Figure 5 shows an absence of suppression of specific cytotoxicity nine days after groups of mice were treated in vivo with 50 mg/kg CB at any time before or after tumor challenge when compared to vehicle-treated controls.

Figures 6, 7 and 8 shows the reversal of immunosuppression produced by adding IL-2 after mice were treated with 50 mg/kg of CB at 19 hours, 3 hours and 72 hours before splenectomy.

Figure 9 compares the cytotoxicity exhibited by spleen cells which are washed prior to treatment with rIl-2 with the cytotoxicity exhibited by unwashed spleen cells treated with rIl-2.

Figure 10 compares the immunosuppression produced by high doses of CB administered in microcapsules with the immunosuppression produced by CB at doses varying between 2 mg/kg to 50 mg/kg administered IP.

Figure 11 shows the effect of treatment with CB at various times before and after tumor challenge on tumor-induced splenic enlargement.

Figure 12 shows the effect of treatment with CB at various times before and after tumor challenge on tumor-induced splenic cellularity.

Brief Description of the Invention

The present invention relates to pharmaceutical compositions and therapeutic methods for treating mammals, especially humans, with cytochalasins to reversibly suppress the heightened immune response (hyperimmunity) in autoimmune disease states or following organ transplants or tissue grafts. In the immunosuppressive method of the present invention, an immunosuppressive dose comprising no less than about one fifth the maximum tolerated dosage of a cytochalasin is administered intravenously, intramuscularly, subcutaneously or orally to a patient suffering from hyperimmunity.

The present invention also relates to intramuscular, intravenous, subcutaneous and oral dosages of cytochalasins which may be useful for inhibiting the growth and metastasis of tumors without producing concomitant immunosuppressive effects. In this aspect of the present invention, cytochalasins comprising no greater than about one fifth the maximum tolerated dosage for a particular route of administration are formulated and administered alone or in combination with other antineoplastic agents for the inhibition of the growth of tumors.

The present invention also relates to the discovery that IL-2 as well as other lymphokines may be used to eliminate the immunosuppressive effects produced by the cytochalasins. In this aspect of the present invention, pharmaceutical compositions and therapeutic methods employing cytochalasins and IL-2 in combination are presented. Optionally, an additional antineoplastic agent other than a cytochalasin may be included for administration with the cytochalasin and IL-2.

The present invention also relates to pharmaceutical compositions and therapeutic methods utilizing cytochalasins in liposomes or

microcapsules to provide sustained release formulations of cytochalasin which deliver large antineoplastic doses of cytochalasin to the site of a tumor without producing concomitant immunosuppressive effects. A surprising discovery of the present invention is that concentrations of cytochalasin up to about three times the maximum therapeutic dosage may be administered in vivo in liposomes or microcapsules without producing the immunosuppressive effects which exist when cytochalasins are not administered in liposomes or microcapsules.

The present invention further relates to semi-synthetic cytochalasin analogues, pharmaceutical compositions containing these analogues and therapeutic methods utilizing these analogues which do not readily metabolize to the enedione system of cytachalasin A (CA).

Detailed Description of the Invention

The following abbreviations and definitions will be employed throughout the specification:

BID - administered twice daily
CA - cytochalasin A
CB - cytochalasin B
CD - cytochalasin D
CE - cytochalasin E
CMC, CM- cellulose - carboxymethyl cellulose
CMC/Tw - carboxymethyl cellulose/Tween
Cytochalasins - synthetic or naturally occurring mold-derived microfilament and membrane-acting compounds.
DMSO - dimethyl sulfoxide
E:T ratio - effector cell:target cell ratio
FAT - vesicles made by a freeze and thaw technique
GI tract - gastrointestinal tract
HPLC - high performance liquid chromatography
IC - inhibitory concentration; IC50 = 50 percent of cells inhibited
IP - intraperitoneal
IV - intravenous

LD - lethal dose
LUV - large unilamellar vesicles
LUVET - LUVs made by the VET technique
MLV - multilamellar vesicles
MTD - maximum tolerated dose
NMR - nuclear magnetic resonance
PBS - phosphate buffered saline
QID - administered four times daily
SC - subcutaneous
SPLV - stable plurilamellar vesicles
TAD - tumor appearance day
TLC - thin layer chromatography
Tw - Tween (polyoxyethylene sorbitan monoalkyl ethers); surface-acting agents
VET - vesicles made by an extrusion technique

The present invention relates to a method for producing transient immunosuppression in mammals using cytochalasins. More specifically, it has been discovered that the cytochalasins may be used as transient immunosuppressive agents for treating hyperimmunity associated with organ transplants, tissue grafts and autoimmune disease, for example rheumatoid arthritis, lupus, autoimmune diabetes, autoimmune thyroiditis, autoimmune hepatitis.

In the immunosuppressive method of the present invention, a cytochalasin is administered to a mammal in an amount within the range of about one fifth the maximum tolerated dose (MTD) to about the MTD for a particular route of administration, preferably about one half to about the MTD of cytochalasin, and most preferably about the MTD. Any of the cytochalasins including cytochalasins A-M as well as the semi-synthetic derivatives of cytochalasin, including 21,22-dihydro-cytochalasin B, 20-deoxy-cytochalasin B, 20-deoxy-21,22-dihydrocytochalasin B, 20-deoxy-20-fluoro-cytochalasin B, 20-deoxy-20-fluoro-21, 22-dihydrocytochalasin B and 21,22-dihydro-cytochalasin A, among others, may be used in the immunosuppressive method of the present invention.

Of course, the numerous cytochalasins differ in their ability to inhibit microfilament formation, phagocytosis, cytokinesis, secretion and exocytosis, and consequently, in their ability to produce immunosuppression. In addition, the route of administration and the pharmacokinetics of the cytochalasin derivative also play an important role in determining the extent of immunosuppression. Generally, however, immunosuppressive dosages of natural and semi-synthetic cytochalasins fall within the range of about one fifth to about the MTD for a particular route of administration.

Depending upon the type of cytochalasin used and the route of administration, the daily dosage of cytochalasin effective for producing transient immunosuppression will generally range from about 0.04 mg/kg to about 150 mg/kg and preferably from about 1.0 mg/kg to about 150 mg/kg. Of course, the concentration of cytochalasin used will be varied according to the route of administration and activity of the cytochalasin. By way of example, for administration of a cytochalasin via subcutaneous injection, the amount of cytochalasin used generally ranges from about 2 mg/kg to about 150mg/kg and preferably 5 mg/kg to about 150 mg/kg. For administration of the cytochalasin via intravenous injection, the amount of cytochalasin used will generally range from about 0.1 mg/kg to about 20 mg/kg and preferably 0.25 mg/kg to about 20 mg/kg. For administration of a cytochalasin via an intramuscular route, the amount of cytochalasin used will generally range from about 1 mg/kg to about 50 mg/kg and preferably about 2.5 mg/kg to about 50 mg/kg. Also by way of example, effective concentrations of CE and CD useful in the present invention will generally be much smaller (about one tenth) than the concentration of CB used because of the difference in potency. Most preferably, the cytochalasins are administered in an amount equal to about the MTD for a particular route of administration. The table presented in figure 1 presents representative MTD for a number of different CB containing vehicles administered to mice IP, SC or IV.

In formulating the cytochalasins for use as immunosuppressive agents, typical pharmaceutical formulation solvents and delivery suspensions may be utilized. The formulations are generally administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. For parenteral administration, the cytochalasins may be used in the form of a sterile, pyrogen-free aqueous solution which may contain other solutes, for example, salts or sugars such as glucose to make the solution isotonic. Any suspension or solvent which is pharmaceutically compatible may be used in the present invention. Of course, the type of suspension or solvent used in the parenteral formulations of the present invention may affect the absorptivity of the cytochalasin. Modifying the formulations to maximize the immunosuppressive effect of the cytochalasins is well within the skill of one of ordinary skill in the formulation arts.

Preferred suspensions for parenteral administration include cytochalasins in CMC 2%/Tween-20 1%. Preferred solutions include cytochalasins in ethanol/saline (1:2) and DMSO.

The immunosuppressive effect of the cytochalasin used may be affected by the site as well as the route of administration. The transient, reversible immunosuppressive effect of the cytochalasins is dependent on timing and the amount of cytochalasin to reach the site of activity. Sites of activity for cytochalasin immunosuppression may include the spleen, bone marrow, lymph nodes, thymus or a transplanted organ or graft site. One of ordinary skill in the art will know to vary the amount, route and site of administration to maximize the concentration of cytochalasin at the site of immunosuppressive activity. When intravenous administration of cytochalasins is contemplated, care must be taken to choose the site of administration to maximize localization of cytochalasin at the site of immunosuppression within a short time frame, i.e., about one to twelve hours after administration, and preferably, one to about three hours after administration.

Immunosuppressive amounts of cytochalasin may also be administered orally in gelatin capsules, powders, syrups, elixirs, aqueous solutions, suspensions and the like. Oral dosage forms are preferably administered as immediate release oral products. An immediate release oral product is one that releases the active agent immediately after the product reaches the gut. It is believed that orally administered cytochalasins which are formulated as immediate release products may reach certain sites of immunosuppressive activity, for example, the spleen, as readily as the parenterally administered cytochalasins, thus promoting the maximum immunosuppressive effect.

The orally administered products may be administered in combination with pharmaceutical carriers and diluents, for example, lactose, sodium citrate, salts of phosphoric acid, magnesium stearate, starch and talc. A preferred route of oral administration is via immediate release soft gelatin capsules in which the cytochalasin is solubilized in a lower molecular weight polyethylene glycol, or another solvent, for example, DMSO, or mixtures thereof, so as to maintain the cytochalasin in a soluble or near-soluble state in the capsule to maximize dissolution, absorptivity and blood concentration of the cytochalasin after the capsule dissolves in the GI tract.

Unlike the immunosuppression produced by traditional immunosuppressive agents or which occur as side effects from the administration of most anti-cancer agents, the immunosuppression produced by cytochalasins is transient and readily reversible. The immunosuppression produced by a bolus dose of cytochalasin generally increases or decreases as a function of the concentration of the cytochalasin at the site of immunosuppressive activity. Therefore, immunosuppression produced by cytochalasins may be carefully controlled with a therapeutic regimen designed to maximize the concentration of cytochalasin at the site of immunosuppression using cytochalasin pharmacokinetic data. Treatment may vary such that an immunosuppressive amount of a cytochalasin may be administered as a bolus dose once or twice daily via a parenteral route, or orally,

once every six to twelve hours (qid or bid). Alternatively, the cytochalasins may be administered at the onset of heightened immunity. Of course, the therapeutic regimen chosen will vary as a function of the activity, route of administration and pharmacokinetics of the cytochalasin chosen.

The present invention also relates to antineoplastic dosage forms of cytochalasin and methods of treatment which inhibit the growth and spread of tumors without producing concomitant immunosuppressive effects. In this aspect of the present invention, a cytochalasin is administered to a patient in an amount equal to no greater than about one fifth of the MTD, and preferably no greater than about one tenth the MTD. As in the immunosuppressive aspect of the present invention, the type, route of administration and pharmacokinetics of the cytochalasin derivative used play an important role in determining the proper dosage. Generally, however, cytochalasin dosages which inhibit the growth and metastasis of tumors but do not produce concomitant immunosuppression fall within the range of about 0.05 mg/kg to about 30 mg/kg.

In this aspect of the present invention, the cytochalasins may be administered parentally or orally in the same manner and using the same solvents and other additives that are used in the immunosuppressive aspect of the present invention, except that lower dosages are to be used to avoid significant immunosuppression. The cytochalasins in this aspect of the present invention may be formulated in combination with certain antineoplastic agents other than cytochalasins; however, it is preferred that these agents should not themselves produce significant immunosuppression unless additional therapy for eliminating immunosuppression is also used.

The present invention also relates to the surprising discovery that sustained release formulations comprising cytochalasins and liposomes and/or microcapsules in dosages significantly higher than those which produce immunosuppression may be administered to patients without causing appreciable immunosuppression. Dosage forms of cytochalasin comprising at least about the maximum

tolerated dosage, and preferably up to about three times the maximum tolerated dosage for a route of administration formulated in liposomes or microcapsules are useful for treating neoplasia without producing immunosuppression. Although numerous antineoplastic agents may be formulated along with the cytochalasin in the liposomes or microcapsules, it is preferred that only those neoplastic agents which exhibit an absence of immunosuppression should be formulated in combination with cytochalasin unless additional therapy for eliminating immunosuppression is also used.

Liposomes may be used as the sustained release delivery vehicle for the administration of the cytochalasins and optionally, an antineoplastic agent. Any of the techniques known in the art for making liposomes may be used in this invention. For example, the original liposome preparation of Bangham et al. (J. Mol. Biol., 1965, 12:238-252) which involves suspending phospholipids in an organic solvent, evaporating the solvent to dryness, adding an appropriate amount of aqueous phase, allowing the mixture to "swell" dispersing the resulting liposomes which consist of multilamellar vesicles (MLVs) by mechanical means. Other techniques involve the use of small sonicated unilamellar vesicles described by Papahadjopoulos et al. (Biochim. Biophys, Acta., 1968, 135:624-638), and large unilamellar vesicles.

Unilamellar vesicles may be produced using an extrusion apparatus by a method described in Cullis et al., PCT Publication No. WO 87/00238, published January 16, 1986, entitled "Extrusion Technique for Producing Unilamellar Vesicles" incorporated herein by reference. Vesicles made by this technique, called LUVETS, are extruded under pressure through a membrane filter. Vesicles may also be made by an extrusion technique through a 200 nm filter; such vesicles are known as VET200s.

Another class of liposomes that may be used are those characterized as having substantially equal lamellar solute distribution. This class of liposomes is denominated as stable plurilamellar vesicles (SPLV) as defined in U.S. Patent No.

4,522,803 to Lenk, et al., monophasic vesicles as described in U.S. Patent No. 4,558,579 to Fountain, et al. and frozen and thawed multilamellar vesicles (FATMLV) wherein the vesicles are exposed to at least one freeze and thaw cycle; this procedure is described in Bally et al., PCT Publication No. 87/00043, January 15, 1987, entitled "Multilamellar Liposomes Having Improved Trapping Efficiencies" which are incorporated herein by reference.

Any of the methods for making liposomes can be used to encapsulate the semi-synthetic or naturally occurring cytochalasins. Pharmaceutical compositions of these liposomal forms of the cytochalasins can be administered in vitro or in vivo as described hereinbelow.

A variety of sterols and their water soluble derivatives have been used to form liposomes; see specifically Janoff et al., U.S. Patent No. 4,721,612, issued January 26, 1988 entitled "Steroidal Liposomes." Mayhew et al. (PCT Publication No. WO 85/00968, published March 14, 1985) described a method for reducing the toxicity of drugs by encapsulating them in liposomes comprising alpha-tocopherol and certain derivatives thereof. Also, a variety of tocopherols and their water soluble derivatives have been used to form liposomes, see Janoff et al., PCT Publication No. 87/02219, April 23, 1987, entitled "Alpha Tocopherol-Based Vesicles."

Alternatively, when an ionizable antineoplastic agent is employed in combination with cytochalasin, the liposomes can be loaded with drug according to the procedures of Bally et al., PCT Publication No. 86/01102, published February 27, 1986, and incorporated herein by reference. This technique allows the loading of antineoplastic agents by creation of a transmembrane concentration gradient across the liposome membranes. This gradient is generated by a concentration gradient for one or more ionic species (e.g., Na^+, Cl^-, K^+, Li^+, or H^+) across the liposome membranes. Preferably, these ionic gradients are pH (H^+) gradients, which drive the uptake of the ionizable antineoplastic agent across

the liposome membranes. Once the liposomes are loaded with the antineoplastic agent(s) by this or any other method, pharmaceutical formulations can be made which can be delivered in vitro or in vivo as described hereinbelow.

In a liposome-drug delivery system, a bioactive agent such as a drug is entrapped in or associated with the liposome and then administered to the patient to be treated. For example, see Rahman et al., U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos et al., U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,114,179; Lenk et al., U.S. Patent No. 4,522,803; and Fountain et al., U.S. Patent No. 4,588,578. In the present invention, as mentioned hereinabove, the semi-synthetic and naturally-occurring mold-derived cytochalasins may be entrapped in or associated with liposomes. Additionally, antineoplastic agents or agents which are administered to eliminate the immunosuppressive effects of the cytochalasins or the additional antineoplastic agents as described hereinabove can be co-entrapped in liposomes, or entrapped in liposomes co-administered with those liposomes containing the cytochalasins. Such formulations of liposomes may be administered simultaneously or sequentially. A preferred method of the present invention is to entrap up to three times the MTD of cytochalasin alone or in combination with an effective amount of an additional antineoplastic agent. This formulation is then administered to the patient as a sustained release form without producing substantial immunosuppression.

During preparation of the liposomes, organic solvents may be used to suspend the lipids. Suitable organic solvents for use in the present invention include those with a variety of polarities and dielectric properties, which solubilize the lipids, for example, chloroform, methanol, ethanol, dimethylsulfoxide (DMSO), methylene choloride, and solvent mixtures such as benzene:methanol (70:30), among others. As a result, solutions (mixtures in which the lipids and other components are uniformly distributed throughout) containing the lipids are formed. Solvents are generally chosen on

the basis of their biocompatability, low toxicity, and solubilization abilities. Liposomes containing the pharmaceutical formulations including cytochalasins of the present invention may be used therapeutically in mammals, especially humans, in the treatment of neoplasms which require repeated administration. The sustained release formulations utilize up to three times the MTD of cytochalasin. Such sustained release compositions are effective against neoplasia without producing substantial concomitant immunosuppression.

High dosages of cytochalasins may also be administered as sustained release formulations in microcapsules without producing immunosuppression. A preferred type of microcapsule is that encapsulated in a film-forming polymer made of a mixture of polylactate-glycolate in a weight ratio of about 45:55 to about 55:45, preferably 50:50, and similar materials as taught by U.S. Patent Nos. 4,675,189, 4,585,482, 4,542,025, 4,530,840, European Patent Applications 87309286.0 (Publication Number 0,266,119), 87307115.3 (Publication Number 0257915), 81305426.9 (Publication Number 0052510) and 83303605.6 (Publication Number 0129619). Other formulations well known in the art may also be used in formulating sustained release versions of cytochalasin in microcapsules. Still other sustained release formulations readily recognized in the art may be used in this aspect of the present invention provided that the total amount of cytochalasin included within the formulation does not produce immunosuppression, i.e., is no greater than about three times the MTD of cytochalasin. It is recognized that a large number of sustained release formulations containing cytochalasin may be used provided that the release of cytochalasin is such that otherwise immunosuppressive levels of cytochalasin are not reached rapidly at immunosuppressive sites, i.e., within a period of at least about 1 to 12 hours, and preferably within a period of at least about 1 to 24 hours.

Delivery systems other than liposomes or microcapsules may also be used for administering both natural and semi-synthetic cytochalasins as immunosuppressive or antineoplastic agents. Such

delivery systems include for example osmotic pumps, transdermal patches, infusion pumps, biodegradable polymers, monoclonal antibody-linked systems, suppositories, rhinile, dragees and troches, among others.

The present invention also relates to the use of IL-2 to eliminate the immunosuppression produced by cytochalasins. It has been discovered that the use of IL-2 will reverse the immunosuppressive effects of cytochalasins, providing an effective means of overcoming the immunosuppression that occurs when high doses of cytochalasins alone, or in combination with other antineoplastic agents, are administered for the treatment of neoplasia. In this aspect of the present invention, the administration of IL-2 to cytochalasin immunosuppressed lymphocytes will eliminate the immunosuppression.

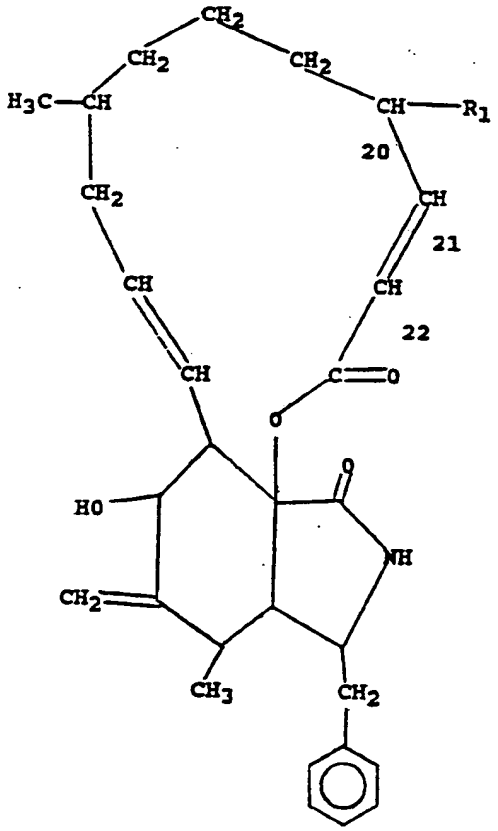
Although IL-2 administered any time during immunosuppression will eliminate the immunosuppression, to maximize its effect, it is preferred that the IL-2 should be administered in conjunction with cytochalasin or within a short period of time thereafter, for example, within one hour. Although the amount of IL-2 administered will vary depending upon the individual patient, the type of cytochalasin used and the extent of immunosuppression, preferred dosages of IL-2 range from about 5,000 to about 15,000 units per kilogram per day. Although IL-2 from any source may be utilized in this aspect of the present invention, including IL-2 produced by cultivating human peripheral blood lymphocytes or other IL-2 producing cell lines, the preferred IL-2 is human recombinant IL-2 (rIL-2, available from DuPont Wilmington, Delaware). It is to be recognized that any protein having IL-2 activity may be used to eliminate the immunosuppressive effects of the cytochalasins and that the term IL-2 includes proteins in which one or more of the amino acids of IL-2 have been changed but in which activity is substantially the same as IL-2. For treatment of immunosuppression in humans, human rIL-2 is preferred.

In cases where the therapeutic method of cytochalasin immunosuppression does not rely purely on the transient reversibility of cytochalasin immunosuppresion for immune restoration, the administration of IL-2 in appropriate concentrations may be used to restore immunity. Alternatively, the administration of IL-2 may be used to regulate the immunosuppressive activity of administered cytochalasin. Such administration may occur during or after the administration of cytochalasin alone or in combination with other antineoplastic agents.

IL-2 may be made by cultivating human peripheral blood lymphocytes (PBL) or other IL-2 producing cell lines. IL-2 may also be made by recombinant DNA technology, which has afforded a means to produce muteins and other modified versions of naturally occurring IL-2 which may be used to practice the present invention. A preferred IL-2, human rIL-2 may be purchased from a number of suppliers (Cetus Corp., Emeryville, Calif.).

IL-2 may be formulated with cytochalasin using any of the solvents, additives and methods described hereinabove, including liposomes and microcapsules for sustained release formulations, provided that the formulations do not disturb the integrity, stability or activity of the IL-2. As explained, IL-2 may be administered in combination with cytochalasin or preferably, shortly thereafter, via parenteral routes of administration, preferably via IV infusion. IL-2 and cytochalasin may also be administered in combination with at least one additional antineoplastic agent. Any number of antineoplastic agents may be used in combination with cytochalasins and IL-2, including doxorubicin, daunorubicin or epirubicin, pyrrolizidine alkaloids, the vinca alkaloids such as vinblastine or vincristine, the purine or pyrimidine derivatives for example 5-fluorouracil, among others, the alkylating agents such as mitoxanthrone, mechlorethamine hydrochloride or cyclophosphamide, platinum compounds such as cis-platinum, folic acid analogs such as methotrexate, and the antineoplastic antibiotics such as mitomycin or bleomycin, among others. Any number of the previously described solvents, additives and methods may be used for formulating such products.

The present invention also relates to a method and formulations for treating splenomegaly (enlarged spleen) in humans. The same formulations of cytochalasin including the same amounts of cytochalasin used to induce immunosuppression are effective for treating splenomegaly resulting from a hyperimmune state. Another aspect of the present invention relates to novel semi-synthetic analogues of CB which are not as readily metabolized to the enedione system of CA as is CB. Synthetic analogues of the present invention have the general structure



where R1 is hydrogen or fluorine, and where the carbon-carbon bond alpha, beta to R1 at C21, C22 is oxidized or reduced. The semi-synthetic cytochalasin analogues of the present invention are commonly known as 20-deoxy-cytochalasin, 20-deoxy-21,21-dihydrocytochalasin, 20-deoxy-20-fluoro-cytochalasin and 20-deoxy-20-fluoro-21,22-dihydrocytochalasin.

The semi-synthetic cytochalasins of the present invention, unlike CB, may not be as readily metabolized to the enedione system

of CA, a system which is believed to be responsible for much of the toxicity associated with the administration of CB and CA. The enedione system may irreversibly bind with any number of thiol groups and other nucleophiles in the biological system, thus producing toxic effects. The semi-synthetic cytochalasins of the present invention are designed to maintain the activity associated with other cytochalasins such as CB, including immunosuppression, antineoplasia and anti-metastasis without readily metabolizing or converting, in vivo to the enedione system of CA.

The semi-synthetic cytochalasins of the present invention are advantageously longer-acting than CB or CA and are designed to reduce the marked deleterious side-effects associated with the administration of CB or CA. The semi-synthetic analogues of the present invention may be made by a number of procedures including total chemical synthesis. However, the preferred route of synthesizing these analogues is to chemically modify CA which has been previously isolated from the mold D. dematioidea (ATCC 24346) or which has been prepared by the oxidation of CB to CA using standard prior art methods. Any of the known prior art methods for isolating CA or CB may be used, but it is preferred to utilize a batch absorption technique according to the procedure set forth in PCT Publication No. WO 88/ 10259, Application No. PCT/US88/02095, published December 29, 1988, entitled "Cytochalasin Purification Methods and Compositions", which is incorporated by reference herein. Such a procedure results in sufficient production of CB or CA to provide starting material for synthetic modifications to produce compositions of the present invention. Modification of CA or CB proceeds by following synthetic methods readily available in the art. The following general methods are provided by way of example only and it is recognized that the semi-synthetic cytochalasins of the present invention may be produced through a number of synthetic pathways. After isolation from mold or alternatively, after oxidation of CB to CA, to produce 20-deoxycytochalasin, CA is subjected to a blocking procedure in which the free hydoxyl group is blocked with, for example, a formyl group (85% HCOOH at 60 C for one hour). After blocking the free hydroxyl group of CA, the 20 keto group is subjected to a reduction

to produce a hydroxyl at the C20 position. The free hydroxyl at C20 is then activated with toluensulfonylchloride to produce the "tosyl" group at C20. After the "tosyl" group is formed, it is displaced with sodium iodide to form the 20-iodo hydroxy blocked cytochalasin. This analogue is then subjected to NaBH4 reduction in solvent and the formyl group is cleaved off the hydroxyl group to produce 20 deoxycytochalasin.

To produce 20-deoxy-21,22-dihydrocytochalasin, the free hydroxyl group of CA is blocked with the formyl group, and the resulting product is subjected to a blocking procedure in which the 20-keto group is blocked as the ketal with, for example 1,3-dihydroxypropane in acid. The activated double bond at the 21,22 position is then reduced in NaBH4 to produce the 21,22 dihydro derivative. After the reduction step, and after removing the blocking group to form the 20 keto group, the 20 keto group may be reduced to form 21,22-dihydrocytochalasin B. Alternatively, the free hydroxyl group may be first blocked with a formyl group and the 20 keto group reduced. The resulting free hydroxyl group is tosylated and the tosyl group displaced with iodine as described hereinabove to produce 20-iodo-21,22-dihydrocytochalasin. 20-iodo-21,22-dihydrocytochalasin may be converted to 20-deoxy-20-fluorocytochalasin by simple displacement of the iodo group with fluorine (KF or CsF/18-Crown 6 in acetonitrile). The 20-fluoro cytochalasin derivative may be a diastereomeric mixture (racemic at C20), but the fluoro group at C20 is preferably of the same configuration as is the C20 hydroxyl of CB.

To produce 20-deoxy-20-fluorocytochalasin, the hydroxyl group of CA is blocked with the formyl group, the 20 keto group reduced, tosylated and iodinated as described above. The 20-iodocytochalasin is then converted to 20-deoxy-20-fluorocytochalsin by simple displacement of the iodo group at C20 with fluorine as described above. The 20-fluoro cytochalasin mixture may be a diastereomeric mixture (the fluorine at C20 is a racemic mixture), but the fluoro group at C20 is preferably of the same configuration as is the C20 hydroxyl of CB. As with the other cytochalasins, the semi-synthetic

cytochalasins of the present invention may be used as immunosuppressive agents or agents for treating neoplasia with or without IL-2 or additional antineoplastic agents. The dosage that is to be administered to produce transient, reversible immunosupression is the same as for the other cytochalasins, i.e., at least about one half the MTD for a particular route of administration. Because of their expected longer duration activity, smaller amounts of semi-synthetic cytochalasins than CB are to be used to produced immunosuppression or to treat neoplasia.

The dosage of semi-synthetic cytochalasin for treating neoplasia preferably ranges from about one tenth the MTD to about one fifth the MTD when cytochalasin is to be administered alone or in combination with additional antineoplastic agents. To minimize or eliminate immunosuppression produced by cytochalasins administered at high, immunosuppressive dosage levels, IL-2 may be administered in combination with the semi-synthetic cytochalasins. The semi-synthetic cytochalasins may be administered via intravenous, intramuscular, sub-cutaneous and oral routes, according to the general principles and methods and using the additives and solvents fully described hereinabove. Sustained release formulations comprising very high dosages, e.g., within the range of about the MTD to about three times the MTD of the semi-synthetic cytochalasins of the present invention and liposomes or microcapsules are also contemplated by the present invention. Such dosages in sustained release form provide sufficient antineoplastic amounts of cytochalasin and produce surprisingly low amounts of immunosuppression.

For purposes of practicing the various aspects of the present invention, it is to be understood that in the treatment of neoplasia or an autoimmune disease state using the pharmaceutical compositions of the present invention the prescribing physician will ultimately determine the appropriate dosage of the cytochalasin and other agents including IL-2 or neoplastic agents for a given human subject and condition, and this can be expected to vary according to the age, weight, and response of the individual as well as the nature

and severity of the patient's disease or condition. Dosages would be ultimately determined by the administering physician according to the specific cytochalasin used, the circumstances of treatment and the pharmacokinetics of the agent in the patient. The general dosage ranges provided herein should provide a guideline to follow in making the final dosage determinations. The dosage of the drug in liposomal or microcapsule form for the treatment of neoplasia without
immunosuppression will generally be up to about three times that employed for the free drug. In some cases, however, it may be necessary to administer dosages outside these limits.

The following examples are provided for purposes of illustrating the present invention. These examples are not to be construed as a limitation on the scope of the invention.

Example 1
Cytochalasin Immunosuppression

GENERAL MATERIALS AND METHODS

CB was extracted and purified from cultures of the mold D. dematioidea (ATCC 24346) utilizing a batch absorption technique according to the procedure set forth in PCT Application PCT/US88/02095). Crystalline CB was subjected to final purification by preparative HPLC (75:25 MeOH/H2O on reverse phase C18 DYNAMAXtm silica) followed by recrystallization from CHCl3. Purity was shown to be greater than 99% by analytical TLC and HPLC.

DBA/2 and C57BL/6 female mice were purchased from Charles River Laboratories, Wilmington, Massachusetts through the Animal Genetics Branch of the National Cancer Institute and used at 8 to 12 weeks of age.

CB was prepared for injection using emulsifying needles of

decreasing bore diameter from 20 gauge to a final 25 gauge according to the method described in Bosquet, et al. submitted for publication. Briefly, suspensions were achieved by weighing CB into a syringe, loading the vehicle into a second syringe, and connecting the syringes with a double-hubbed emulsifying needle.

The suspension was forced back and forth repeatedly through the coupling tube until flow was unimpeded, then progressively the bore diameter was decreased from 21 gauge, to 23 gauge and finally to 25 gauge. Syringes and couplers were washed with MeOH and the MeOh wash analyzed on TLC to determine residual CB that was not actually delivered to the test animals.

The culture media for cell culture was RPMI 1640 medium with HEPES, fetal bovine serum, sodium pyruvate, gentamycin, penicillin/streptomycin, MEM non-essential amino acids and trypan blue, purchased from GIBCO, Grand Island, New York, USA.

RPMI 1640 medium with HEPES was supplemented for the 4-day sensitization cell cultures with 10% fetal bovine serum, 0.1 mg/ml gentamycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% glutamine, and 50 uM mercaptoethanol. Medium was immediately sterilized by membrane filtration and used immediately or, if not used immediately, was directly sterilized prior to use. RPMI 1640 medium used for the 4 hour Cr cytotoxicity assay was supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin solution.

P815 mastocytoma tumor cells were maintained in ascites form by weekly intraperitoneal passage into syngeneic DBA/2 mice. Tumor cells used for antigen in 4-day sensitization assays were harvested 72 hours after passage of 5 to 10 x 106 cells. The cells were washed, resuspended in RPMI 1640 medium with HEPES buffer and x-irradiated on ice in a 35 X 10 mm tissue culture dish at a rate of 200 rad/min and 10 cm target distance for a total of 21 minutes (4200 rad). Cr51 labelled tumor used for targets in a 4 hour cytotoxicity (LMC) assay were prepared according to the method of Cerottini, et al. (1972 Nature, 237, 272 and 1974 J. Exp. Med., 140,

703) with the modifications of Orsini, et al. (1977, Cancer Res., 37, 1719).

For in vivo allogeneic challenge, 3 X 107 P815 mastocytoma tumor cells were implanted IP into C57BL/6 mice previously treated or to be treated with IP CB at concentrations of 50 mg/kg or with vehicle (CMC/TW- control). The mice were injected with this dose at -24, -12. -6, -3, simultaneously with, or 24, 48 or 72 hours after implantation. 9 days after tumor challenge, splenectomy occurred. In experiments where effects on resistance to allogeneic challenge were to be monitored for an extended period, treated animals were maintained for fifty days.

For in vitro analysis of CB effects, spleens were aseptically removed from drug-treated and vehicle-treated (control) mice at 72 hours, 19 hours or three hours after injection with CB. Spleen cells were then forced through coarse (50 mesh) and fine (200 mesh) sterilized stainless steel wire mesh to make a single cell suspension. Cells were washed three times (except where indicated) with RPMI 1640 medium and viability was determined by trypan blue exclusion.

The 4-day sensitization of splenic lymphocytes in the presence of x-irradiated P815 tumor antigen was carried out by the method of Cerottini, et al. (1972 Nature, 237, 272 and 1974 J. Exp. Med., 140, 703) with the modifications of Orsini, et al. (1977, Cancer Res., 37, 1719). Spleen cells were cultured in 17.8 X 35 mm 6- well tissue culture plates (Costar, Cambridge, Mass., USA) in a total volume of 2 ml. Each well contained 2 X 107 viable spleen cells and 4 X 105 x-irradiated P815 cells. At the end of the 4 day culture period effector lymphocytes were recovered from each well by gentle scraping with a rubber policemen, and triplicate cultures were pooled. Cells were washed with RPMI 1640 medium and counted with a hemacytometer or flow cytometer (Coulter Diagnostics, Hialeah, Fla., USA). The cytotoxic activity of sensitized spleen cells recovered from the 4 day cultrures was determined in a standard 4 hour 51Cr release assay as previously described by Bogyo and Mihich, Cancer

Research, 40, 650 (1980). Each cell preparation was assayed with a minimum of four different effector:target cell ratios ranging from 100:1 to 6:1. The percentage of 51Cr release was calculated according to the formula:

$$\text{51Cr release} = \frac{\text{cpm supernatant} \times 100}{\text{cpm supernatant} + \text{cpm pellet}} \times 100$$

Later results replaced the formula denominator with maximum release of 51Cr from cultures treated with 1% SDS.???????? The percentage of specific 51Cr release represents values obtained with immune effectors minus values obtained with control lymphocytes cultured for 4 days in the absence of P815 antigen. Administration of 50 mg/kg CB IP 19 hours before removal of splenic lymphocytes in 4 experiments consistently impaired the development of cytotoxic T cells in the allogeneic sensitization assay. The range of inhibition observed was from 33.2% to 56.6% (E:T ratio = 25/1).

When CB was administered IP at 19 hours and 3 hours before splenectomy, decreasing, but some inhibition was produced after the administration of 25 mg/kg and 10 mg/kg as shown in Figures 2 (19 hours) and 3 (3 hours). Two experiments using 5 mg/kg CB failed to show significant immunosuppression. 50 mg/kg CB was administered IP at 3 hours and 72 hours before splenic lymphocytes were removed for sensitization cultures. The range of inhibition at 3 hours before splenectomy was 57.0% to almost 100% as shown in Fig. 3. CB administered 72 hours before splenectomy failed to produce consistent immunosupression (data not shown).

Example 2

Responsiveness In Vivo to Allogeneic Tumor Challenge in CB-treated Mice

Effect of CB at the maximum tolerated IP bolus dose on allogeneic anti-tumor responsiveness was tested directly in vivo.

C57Bl/6 mice treated with CB at 50 mg/kg IP as a bolus suspension were challenged with 3 X 107 viable P815 mastocytoma cells IP at various times before and after CB treatment. Animals were monitored for their ability to reject the allogeneic tumor challenge and for specific cytotoxicity in spleens taken 9 days after tumor challenge. Figure 4 shows the effect of the IP administration of 50 mg/kg on the allogeneic rejection response. Allogeneic responsiveness is compromised when CB is given 12 or 6 hours prior to tumor challenge to the extent that the allogeneicchallenge is lethal in some of the animals. Survivors in these groups appeared to have prolonged allogeneic tumor growth since they showed evidence of abdominal distension and illness up to and beyond day 9 in animals that survived to that point and were maintained for observation beyond day 9. Allogeneic tumor growth was also apparent in animals treated with CB 3 hours prior to tumor challenge even though all animals survived the challenge. The time course of this in vivo suppression thus showed maximum effect between 3 and 12 hours following CB treatment, and is consistent with the effects observed in spleens removed from animals 3 hours after CB treatment and tested for allogeneic responsiveness in vitro.

Spleens sensitized in vivo by challenge with allogeneic tumor and excised from CB-treated and control groups 9 days after tumor challenge were tested for specific allogeneic tumor cell cytotoxicity in vitro. Figure 5 shows no suppression of specific cytotoxicity in any of the groups treated with CB at any time before or after tumor challenge when compared to vehicle-treated controls. In the groups treated with CB either 6 or 3 hours prior to tumor challenge, groups in which allogeneic tumor growth was continuing at the time of spleen excision, specific cytotoxicity was increased beyond control levels.

Example 3

Effects of rIL-2 on Splenic Sensitization

Human recombinant interleukin-2 (obtained as a gift from DuPont

Corp, Wilmington, Del., USA) was added to unwashed cultured splenic cells from example 1 at a level of 10 U/ml in 100 microliters of RPMI 1640 medium at the time of culture initiation. Unwashed and washed spleen cells were used in order to determine whether restoration of immune-responsiveness by rIL-2 could be obtained under conditions where any endogenous CB, CB metabolites or suppressive factors remained present. At the end of the 4 day culture period effector lymphocytes were recovered from each well by scraping. The cells were washed and counted with the hemacytometer and then subjected to the ^{51}Cr release assay as described in Example 1. The addition of 10 U/ml of rIL-2 to the 4 day sensitization cultures consistently reversed the immunosuppression produced by prior in vivo treatment of lymphocytes with CB. This was observed for CB administered at 72 hours, 19 hours and 3 hours before splenectomy for all levels of CB administered as shown in figures 6, 7 and 8. Figure 9 shows that washing the cells before growing the cells in culture results in less immunosuppression when no rIL-2 is added to the culture, but that rIL-2 reversed immunosuppression in cells that were not washed before being grown in culture.

In four experiments in which rIL-2 was added to control lymphocyte cultures without P815 antigen, cytotoxic activity increased between 18.9% and 28.4% (data not shown).

Example 4

Effect of CB on Spleen Weight and
Spleen Cellularity in Unchallenged Spleen Cells

To establish the actual responsiveness per spleen, the effect of CB at doses of 50, 25, 10, and 5 mg/kg IP on spleen weights and on spleen cellularity when spleens were harvested 3 or 19 hours after CB treatment were determined. There was no significant effect of CB treatment on spleen weight, with a range from 92 to 110% of the mean spleen weight of matched vehicle-treated controls and no significant dose-response effect as shown in Table 1 below.

Table 1

EFFECT OF CB IP ON SPLEEN WEIGHTS

CB IP (mg/kg)	Time of Treatment (Hours) -3 n=6	-3 n=3	-19 n=3
	% of Vehicle Controls		
50	96	94	92
25	97	94	107
10	98	ND	101
5	92	ND	110

Example 5

Sustained Release Formula
Effects on Splenic Sensitization

In experiments where microencapsulated CB (formed from a mixture of polylactate-glycolate under contract from Southern Research Institute- which may also be prepared according to the general method taught by U.S. Patent No. 4,389,330) was administered to mice and the animals were retained for observation for prolonged periods there was no evidence of apparent toxocity as evidence by the absence of deaths, no obvious signs of illness and the maximum weight loss averaged 3%. Figure 10 shows that a dose of CB in microencapsulated form which is 3-fold above the maximum tolerated dose for IP bolus administration can be administered to mice without producing significant immunosuppression either 19 hours or 72 hours following injection.

The times used for evaluation of potential immunosuppression by microencapsulated CB were chosen based on a determination of the time course of tissue distribution of the microencapsulated drug. CB was shown to be released from the microcapsules at a rate sufficient to produce its maximum concentration in the spleen and other organs by 3 days after injection (compared to 3 hours by way of 50 mg/kg IP bolus suspension administration). In spite of the high splenic concentration of CB at 3 days following injection with microencapsulated CB, there is only marginal immunosuppression apparent (Figure 10).

This experiment indicates that sustained release formulations of CB and other cytochalasins may be used as antineoplastic agents at higher concentrations than the MTD without producing substantial immunosuppressive effects.

Example 6
CB Effects on In Vivo Lymphocyte Sensitization

Because CB inhibits cytotoxic T cell response to tumor sensitization in vitro, the effects of a single IP administration of CB on in vivo response of splenic lymphocytes to proliferating P815 tumors was observed. A single 50 mg/kg IP bolus of CB did not impair the day 9 or day 10 response of mice injected IP with 3 X 107 P815 cells as shown by the results of a 51Cr cytotoxicity assay in Table 1. This lack of immunosuppression was consistent regardless of whether CB was administered before or after P815 tumor.

Example 7
CB Effects on Splenomegaly

Allogeneic challenge of C57B1/6 mice with P815 mastocytoma cells produces marked splenomegaly in vehicle-treated controls when spleens are excised and weighed 9 days after allogeneic tumor challenge. The increase in spleen weights is more than 3-fold

greater than in animals not challenged with P815 cells. Treatment with CB at various times before and after tumor challenge shows a time-dependent effect on tumor-induced splenic enlargement as shown in Figure 11 and a corresponding effect on splenic cellularity as shown in Figure 12.

Example 8

Synthesis of 20-deoxy-cytochalasin

CA is isolated from the mold D. dematioidea (ATCC 24346) or oxidized from CB isolated from that same mold according to the procedure set forth in PCT Publication No. WO 88/10259. CA is subjected to blocking of the hydroxyl group with a formyl group by stirring CA in an excess of 85% HCOOH at 60 C for one hour or acetylformate/pyridine at -20 C, isolated by ether extraction followed by evaporation of solvent. The crude formyl blocked Cytochalasin A is then subjected to reduction in NaBH4/Dimethylformamide (DMF) or dimethylsulfoxide (DMSO) to produce the formyl blocked derivative having a free hydroxyl group at C20. The free hydroxyl group at C20 is tosylated (excess toluenesulfonylchloride/pyridine at 37 C overnight) and the tosylated product is extracted with ether after the excess pyridine is evaporated (several times with ethanol). The tosylated product is then subjected to reaction with a 1.5 molar excess of NaI in DMF or DMSO to displace the tosyl group with an iodo group at C20, which is then subjected to reductive displacement of the iodo group with NaBH4 in DMF/DMSO and deblocking of the formyl group under acidic conditions (6N HCl)) to produce 20-deoxycytochalasin. We note that the 20-iodocytochalasin derivative may be used to synthesize 20-fluorocytochalasin in Example 9. 20-deoxycytochalasin may be purified from the reaction mixture using preparative HPLC. It is sometimes preferable, but more time consuming, to isolate the intermediates of the synthesis using preparative HPLC (75:25 MeOH/H20 on reverse phase-C18 DYNAMAXtm silica gel columns) before proceeding on to the next synthetic step.

Example 9

Synthesis of 20-deoxy-21,22-dihydrocytochalasin

As in Example 8, the free hydroxyl group of CA is blocked with the formyl group 85% HCOOH at 60 C or acetylformate/pyridine at -20 C, and the resulting product is subjected to a blocking procedure in which the 20-keto group is blocked as the ketal with 1,3-dihydroxypropane or alternatively with 1,3-dimethoxypropane in toluenesulfonic acid. After extraction in ether, the ketal-protected derivative is subjected to NaBH4 reduction (DMF or DMSO) or Pd/Charcoal/H2 reduction in ethanol to produce the ketal protected 21,22 dihydrocytochalasin. The resulting product is then subjected to acid cleavage of the ketal (toluenesulfonic acid in dioxane) followed by blocking of the free hydroxyl group with a formyl group in 85% HCOOH at 60 C for one hour or alternatively, acetylformate/pyridine at -20 C. The 20-keto group is reduced to the hydroxy group, the hydroxy group is tosylated and the tosyl group is displaced by iodination (this iodo intermediate may be used to prepare the product of Example 11) reduced according to the method described in Example 8. The formyl group is cleaved in 6N HCl. The resulting product, 20-deoxy-21,22-dihydrocytochalasin may be purified from the reaction mixture using preparative HPLC (as described in example 8).

Example 10

Synthesis of 20-deoxy-20-fluoro-cytochalasin

20-deoxy-20-fluorocytochalasin may be produced from 20-iodocytochalasin from example 8 by simple displacement of the iodo group at C20 with KF or CsF/18 Crown 6 (1 Molar equivalent of 18 Crown 6) in acetonitrile followed by removal of the formyl blocking group (6N HCl). Preparative HPLC may be performed as described in Example 8.

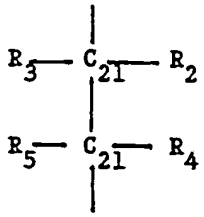
Example 11

Synthesis of 20-deoxy-20-fluoro-21,22-dihydrocytochalasin

20-deoxy-20-fluoro-21,22-dihydrocytochalasin may be produced from 20-iodo-21,22-dihydrocytochalsin from example 9 by simple displacement of the iodo group at C20 with KF or CsF/18 Crown 6 (1 molar equivalent of 18 Crown 6) in acetonitrile as per example 10 followed by removal of the formyl blocking group (6N HCl).

Preparative HPLC may be performed as previously described. It is to be understood that the examples and embodiments described hereinabove are for the purposes of providing a description of the present invention by way of example and are not to be viewed as limiting the present invention in any way. Various modifications or changes that may be made to that described hereinabove by those of ordinary skill in the art are also contemplated by the present invention and are to be included within the spirit and purview of this application and the following claims.

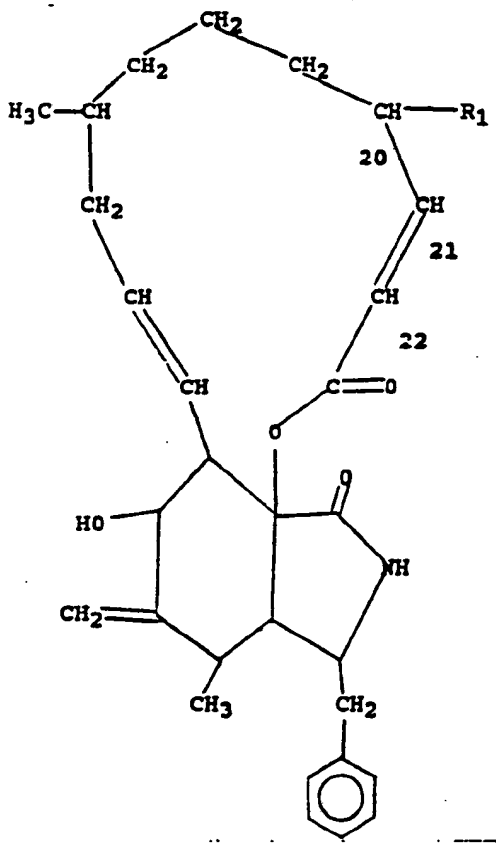
The invention also contemplates new derivatives of cytochalasins as shown at page 22 wherein the formula for the oxidized or reduced C_{21}, C_{22} carbon is



wherein R_2, R_3, R_4 and R_5 are hydrogen, hydroxy or halogen, wherein R_2 and R_3 or R_4 and R_5 taken together are oxygen, or wherein R_2 and R_4 when taken together are oxygen, and additionally wherein R_1 is hydrogen or fluoro.

Claims:

1. A composition of the general structure



where R1 is hydrogen or fluorine and the carbon-carbon bond alpha, beta to R1 at C21,C22 is oxidized or reduced.

2. A pharmaceutical formulation for treating a patient with neoplasia comprising an antineoplastic disease-treating effective amount of a composition from claim 1 and a pharmaceutical carrier or diluent.

3. The composition according to claim 2 adapted for parenteral administration.

4. The composition according to claim 2 adapted for oral administration.

5. A pharmaceutical formulation for treating a mammal suffering from

hyperimmunity comprising a pharmaceutically effective amount of a composition from claim 1 and a carrier or diluent.

6. The formulation according to claim 5 adapted to treat hyperimmunity wherein said composition comprises about one-fifth to about the MTD of said composition for a particular route of administration.

7. The formulation according to claim 5 adapted for parenteral administration.

8. The formulation according to claim 5 adapted for oral administration.

9. A sustained release formulation comprising a composition from claim 1 in an amount ranging from about the MTD to about three times the MTD for a particular route of administration and a sustained release administration vehicle.

10. The sustained release formulation according to claim 9 wherein said vehicle is selected from the group consisting of liposomes and microcapsules.

11. A method of treating a mammal for hyperimmunity in an autoimmune disease state or subsequent to an organ transplant or tissue graft comprising administering an amount of a cytochalasin compound effective for producing immunosuppression.

12. The method according to claim 11 wherein said cytochalasin is administered in an amount equal to about one-fifth the MTD to about the MTD of said cytochalasin for a particular route of administration.

13. The method according to claim 12 wherein said cytochalasin is selected from the group consisting of Cytochalasin A, Cytochalasin B, Cytochalasin D and Cytochalasin E.

14. The method according to claim 13 wherein said cytochalasin is a synthetic cytochalasin selected from the group consisting of the compositions from claim 1.

15. The method according to claim 13 wherein cytochalasin B is administered parenterally in amount ranging from about 10 mg/kg to about 50 mg/kg once or twice daily.

16. The method according to claim 13 wherein said cytochalasin is selected from the group consisting of Cytochalasin D and Cytochalasin E and is administered in an amount ranging from about 1 mg/kg to about 5 mg/kg once or twice daily.

17. A pharmaceutical formulation for treating a patient with neoplasia comprising an antineoplastic disease-treating effective amount of a cytochalasin producing immunosuppression and an amount of interleukin-2 effective to eliminate said immunosuppression.

18. The formulation according to claim 17 wherein said cytochalasin is selected from the group consisting of Cytochalasin A, Cytochalasin B, Cytochalasin D and Cytochalasin E.

19. The formulation according to claim 17 wherein said cytochalasin is selected from at least one of the group consisting of the compositions of claim 1.

20. The formulation according to claim 17 wherein said interleukin-2 comprises about 5,000 units to about 15,000 units per kilogram of said patient.

21. The formulation according to claim 17 further comprising an antineoplastic agent other than a cytochalasin in an amount effective to treat said patient's neoplasia.

22. The formulation according to claim 21 wherein said antineoplastic agent is adriamycin.

23. A method for treating a patient with neoplasia comprising administering the pharmaceutical formulation according to claim 17 to a patient suffering from neoplasia.

24. The method according to claim 23 wherein said cytochalasin is selected from the group consisting of Cytochalasin A, Cytochalasin B, Cytochalasin D and Cytochalasin E.

25. The method according to claim 23 wherein said cytochalasin is selected from at least one of the group consisting of the compositions of claim 1.

26. A method of treating neoplasia comprising administering a cytochalasin in an amount effective to treat neoplasia and produce immunosuppresion and an amount of IL-2 effective to eliminate the immunosuppression.

27. A pharmaceutical formulation for treating neoplasia without producing immunosuppression comprising a cytochalasin in an amount equal to at least the maximum therapeutic dosage effective for treating neoplasia and a pharmaceutical delivery vehicle selected from the group consisting of liposomes and microcapsules.

28. The pharmaceutical formulation according to claim 27 wherein said cytochalasin is selected from the group consisting of Cytochalasin A, Cytochalasin B, Cytochalasin D and Cytochalasin E.

29. The pharmaceutical formulation according to claim 27 comprising cytochalasin B and liposomes.

30. The composition according to claim 27 wherein said cytochalasin comprises at least about the MTD and no greater than three times the MTD for a particular route of administration.

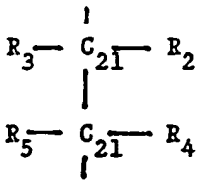
31. A method of treating splenomegaly in a mammal comprising administering an amount of a cytochalasin compound effective to reduce the size and cell number of the spleen.

32. The method according to claim 31 wherein said cytochalasin is administered in an amount ranging from about one half the MTD to about the MTD for for a particular route of administration.

33. The method according to claim 31 wherein said cytochalasin is selected from the group consisting of Cytochalasin A, Cytochalasin B, Cytochalasin D and Cytochalasin E.

34. The method according to claim 31 wherein said cytochalasin is a synthetic cytochalasin selected from the group consisting of the compositions from claim 1.

35. The composition of claim 1 wherein the formula for the oxidized or reduced C_{21}, C_{22} carbon is



wherein R_2, R_3, R_4 and R_5 are hydrogen, hydroxy or halogen, wherein R_2 and R_3 or R_4 and R_5 taken together are oxygen, or wherein R_2 and R_4 when taken together are oxygen.

36. The composition of claim 35 wherein R_1, R_2, R_3, R_4 and R_5 are hydrogen.

37. The composition of claim 36 wherein R_1 is fluoro, and wherein R_2, R_3, R_4 and R_5 are hydrogen.

FIG. 1

CYTOCHALASIN B IN VIVO
MAXIMUM TOLERATED DOSES*: IP, SC, IV

ROUTE	VEHICLE	FORMULATION	MG/KG/DAY
IP	CMC/TWEEN	SUSPENSION	50
	DMSO	SOLUTION	25
SC	CMC/TWEEN	SUSPENSION	150/100
	DMSO	SOLUTION	100
IV	CMC/TWEEN	SUSPENSION	20
	ETOH/SALINE	SOLUTION	20/10/5

* IN B6D2F1, CD2F1, OR C57B1/6 MICE

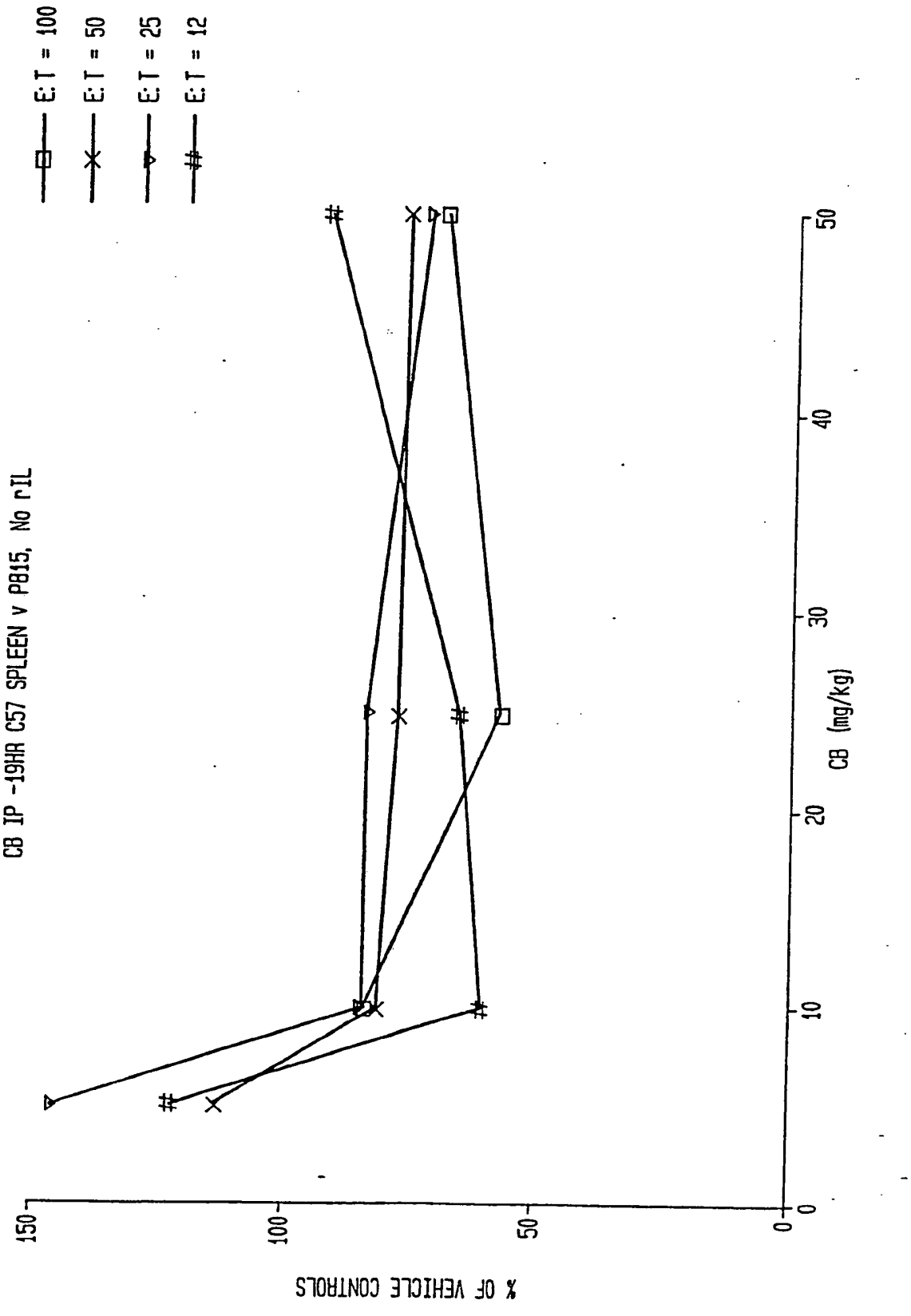


FIG. 2
CB IP -19HR C57 SPLEEN v P815, No rIL

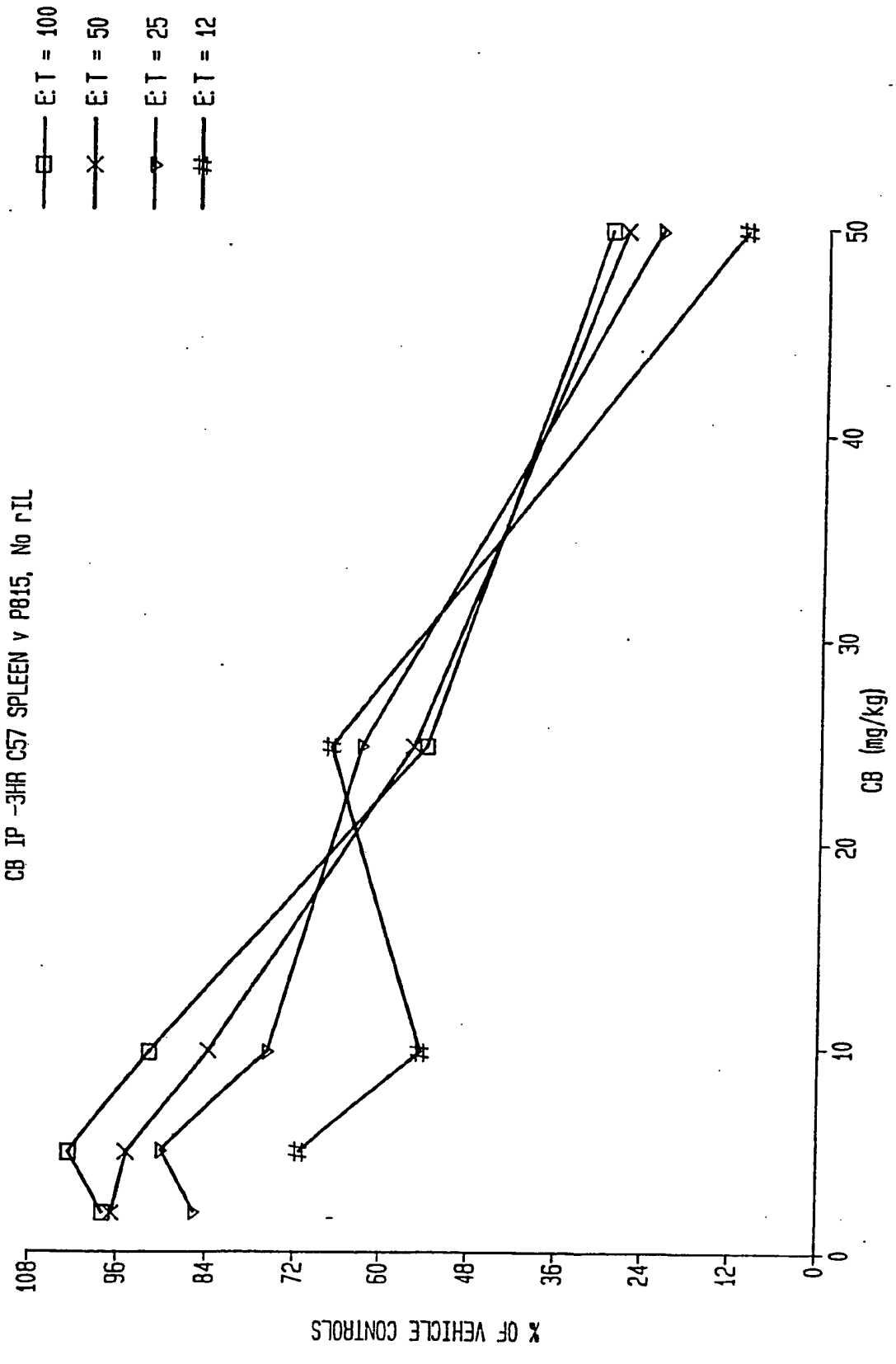

E:T = 100
E:T = 50
E:T = 25
E:T = 12
CB IP -3HR C57 SPLEEN v P815, No rIL
% OF VEHICLE CONTROLS
CB (mg/kg)
108
96
84
72
60
48
36
24
12
0
10
20
30
40
50

FIG. 3

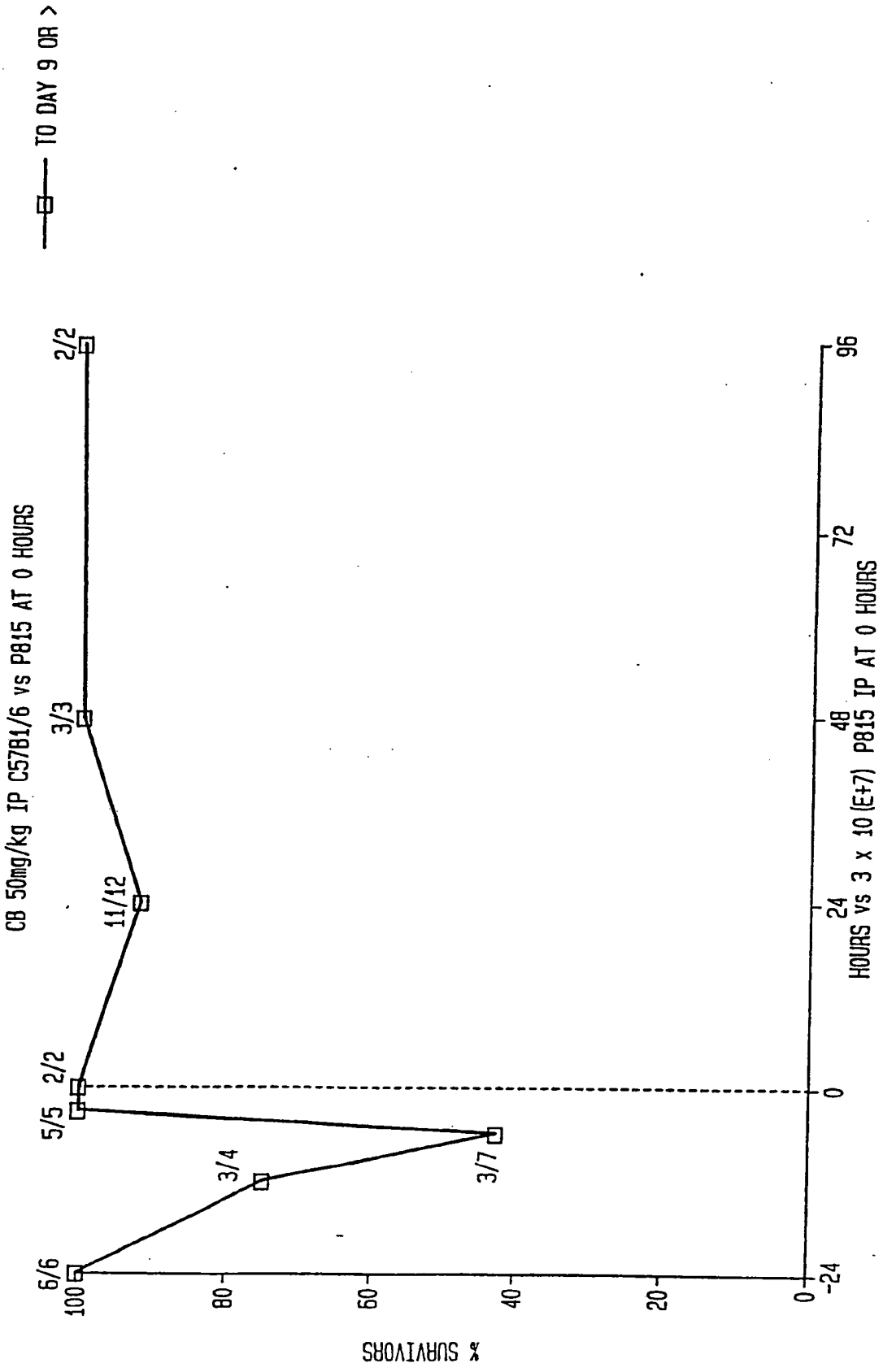

TO DAY 9 OR >
CB 50mg/kg IP C57B1/6 vs P815 AT 0 HOURS
% SURVIVORS
HOURS vs 3 x 10 (E+7) P815 IP AT 0 HOURS
6/6
3/4
5/5
2/2
11/12
3/3
2/2
3/7
100
80
60
40
20
0
-24
0
24
48
72
96

FIG. 4

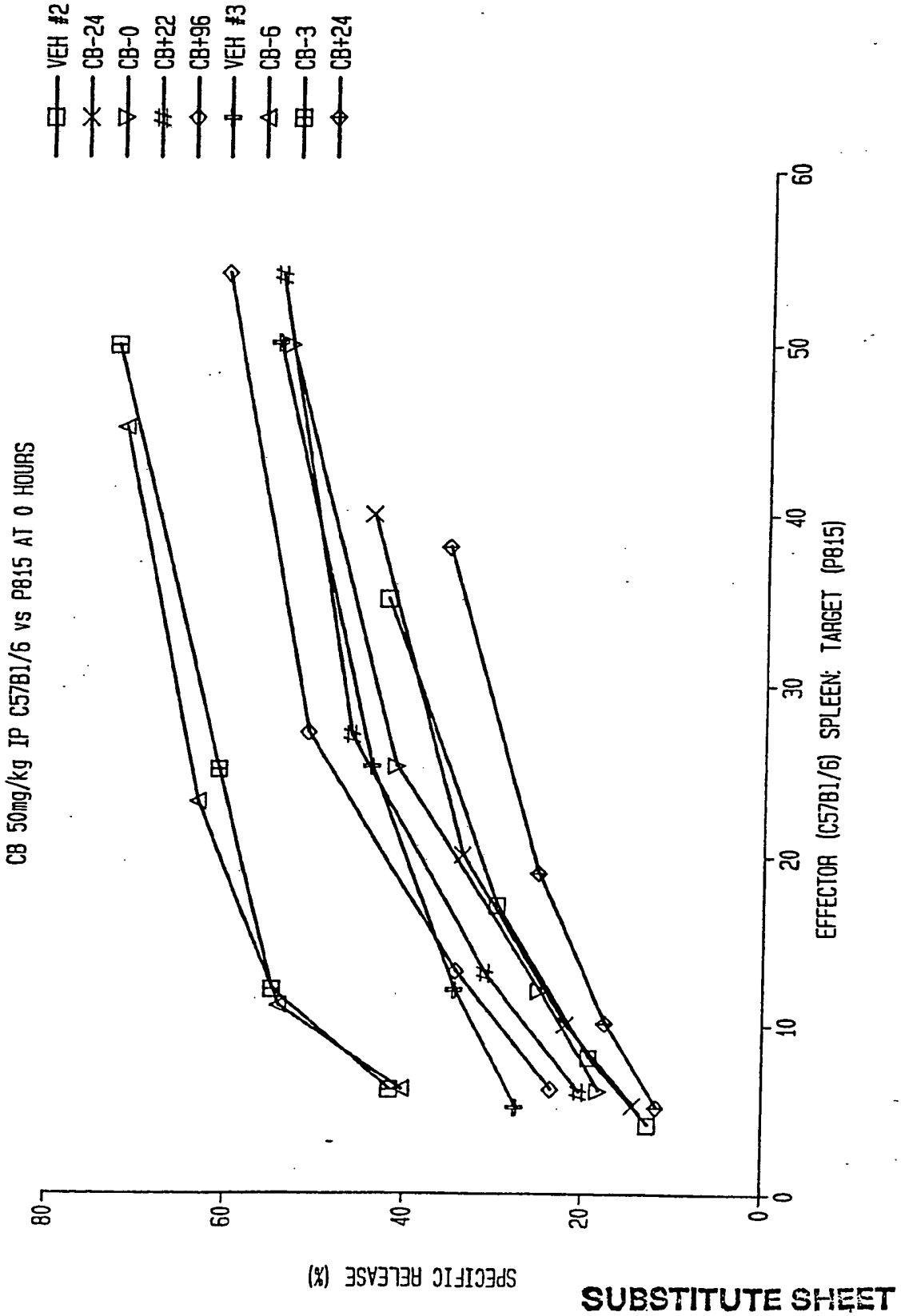


FIG. 5
CB 50mg/kg IP C57B1/6 vs P815 AT 0 HOURS

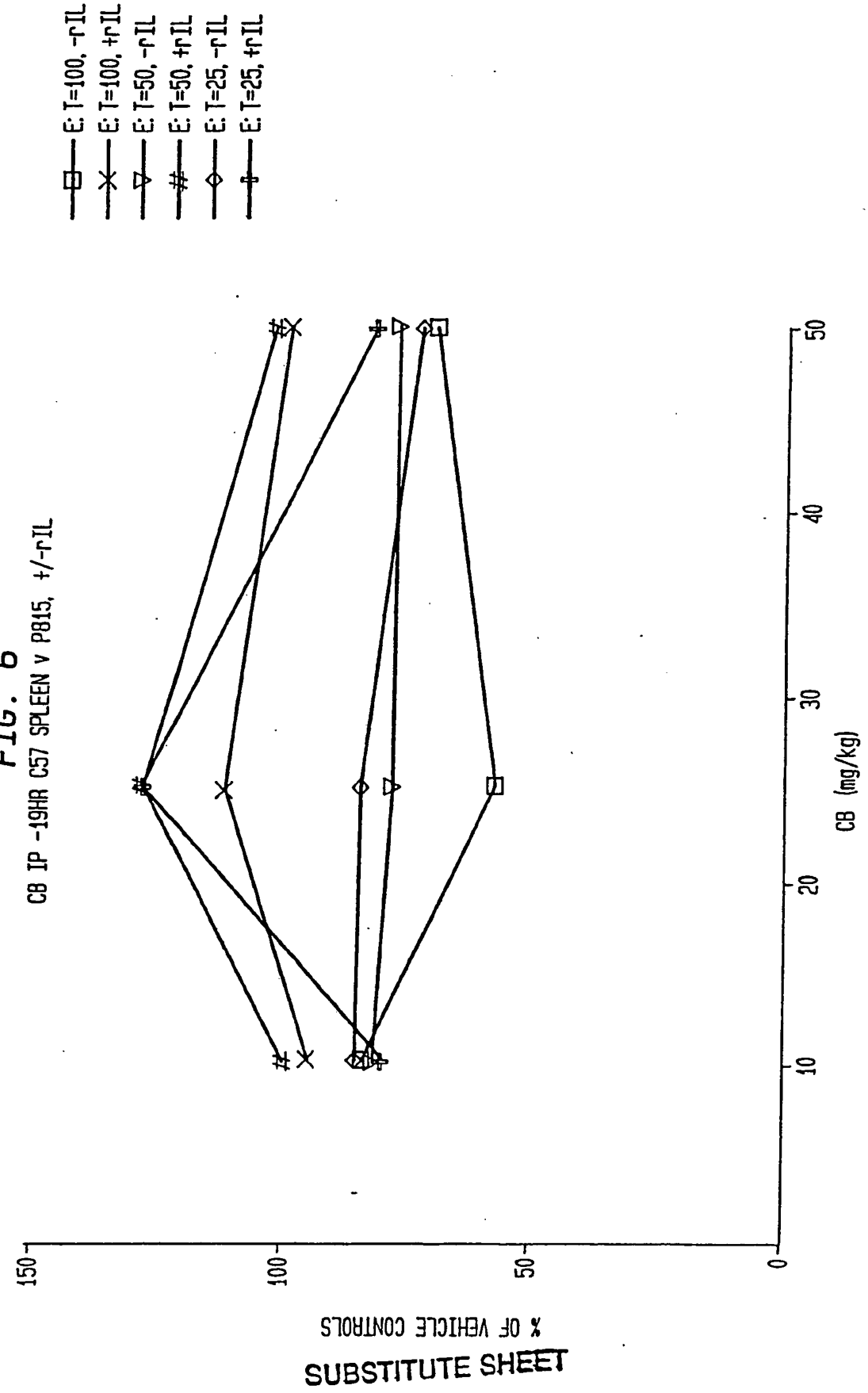

E: I=100, -rIL
E: I=100, +rIL
E: I=50, -rIL
E: I=50, +rIL
E: I=25, -rIL
E: I=25, +rIL
CB IP -19HR C57 SPLEEN v P815, +/-rIL
% OF VEHICLE CONTROLS
150
100
50
0
10
20
30
40
50
CB (mg/kg)

FIG. 6

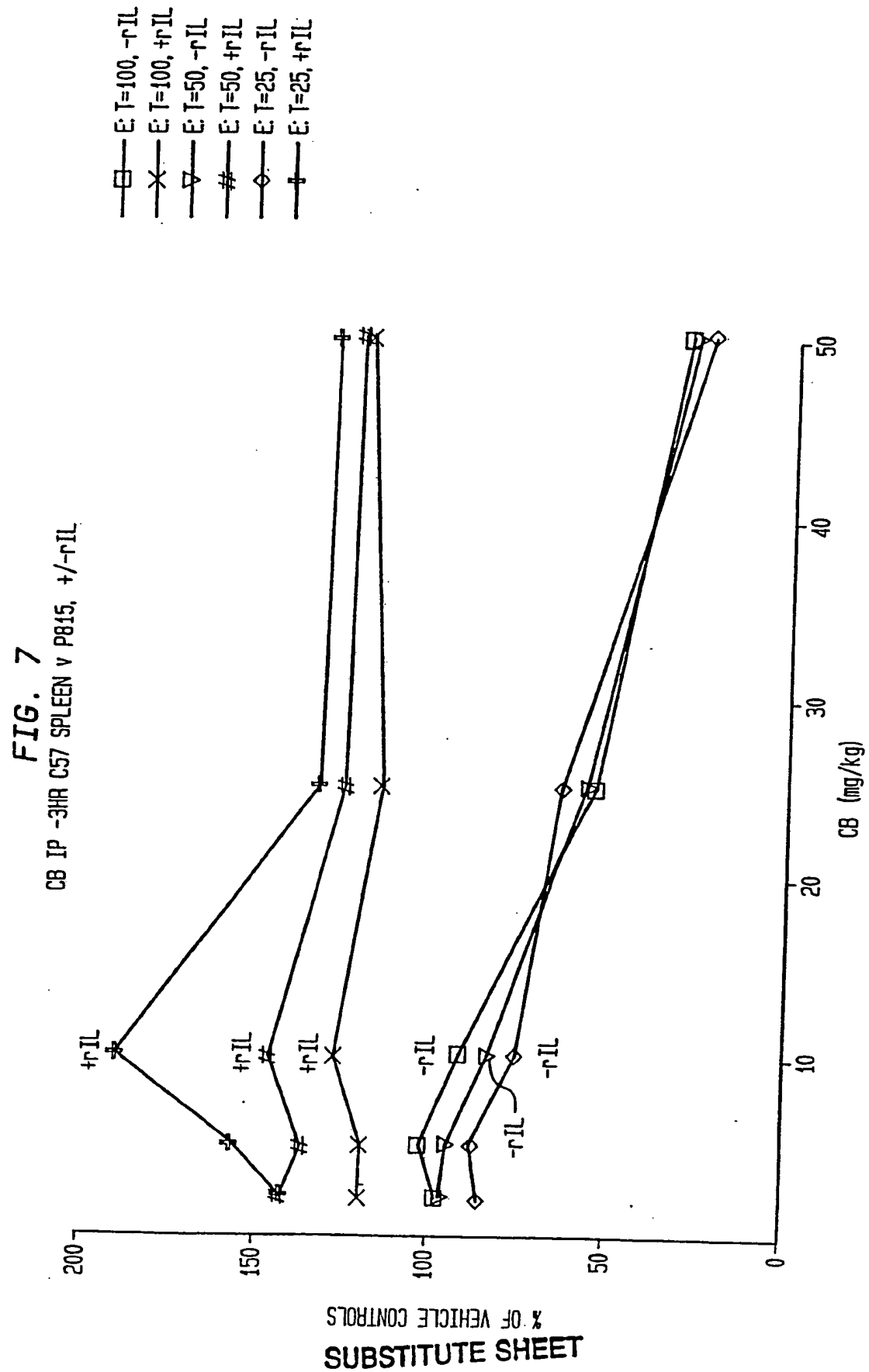


FIG. 7
CB IP -3HR C57 SPLEEN v P815, +/-rIL

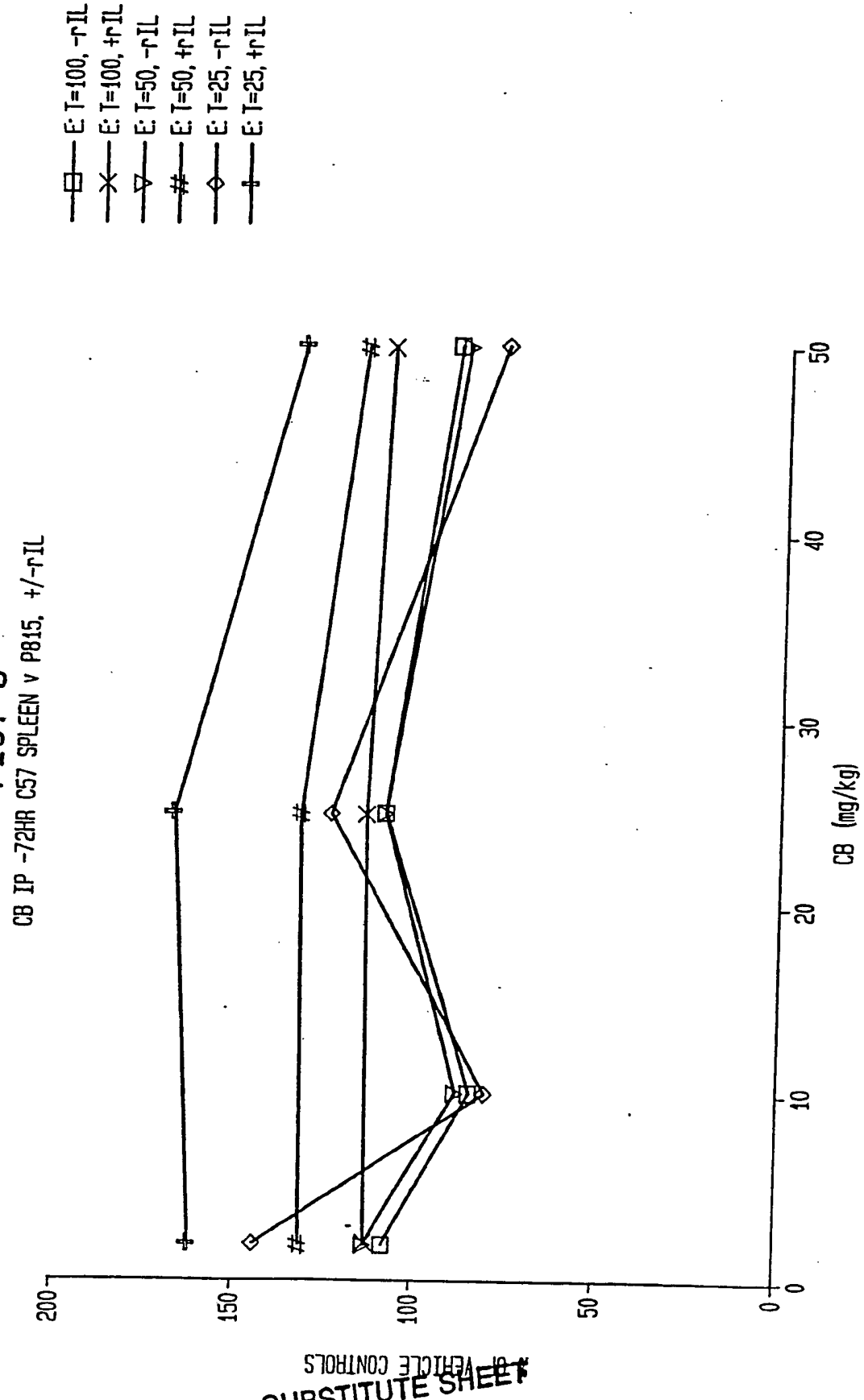


FIG. 8
CB IP -72HR C57 SPLEEN v P815, +/-rIL

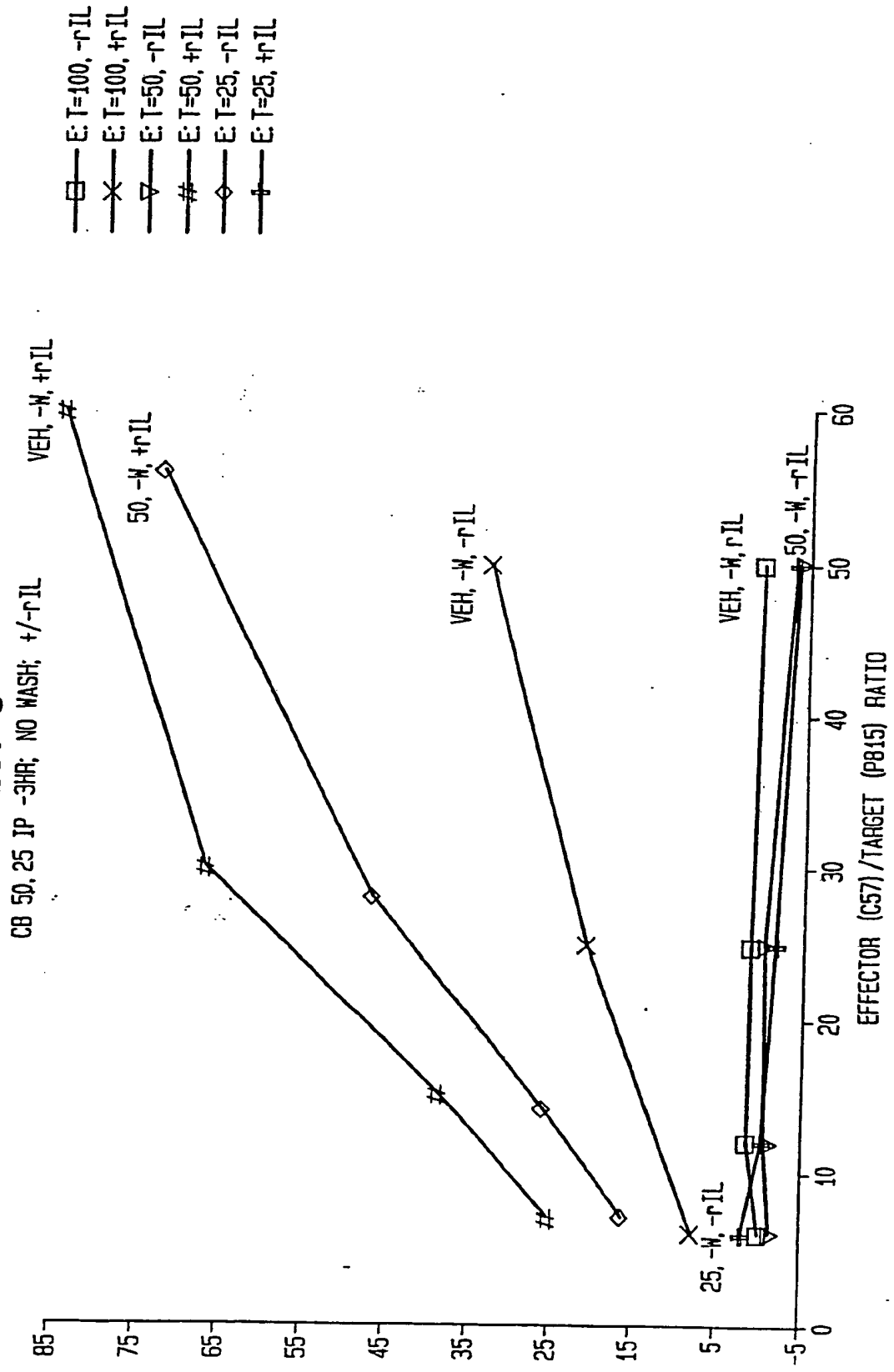


FIG. 9
CB 50, 25 IP -3HR; NO WASH; +/-rIL

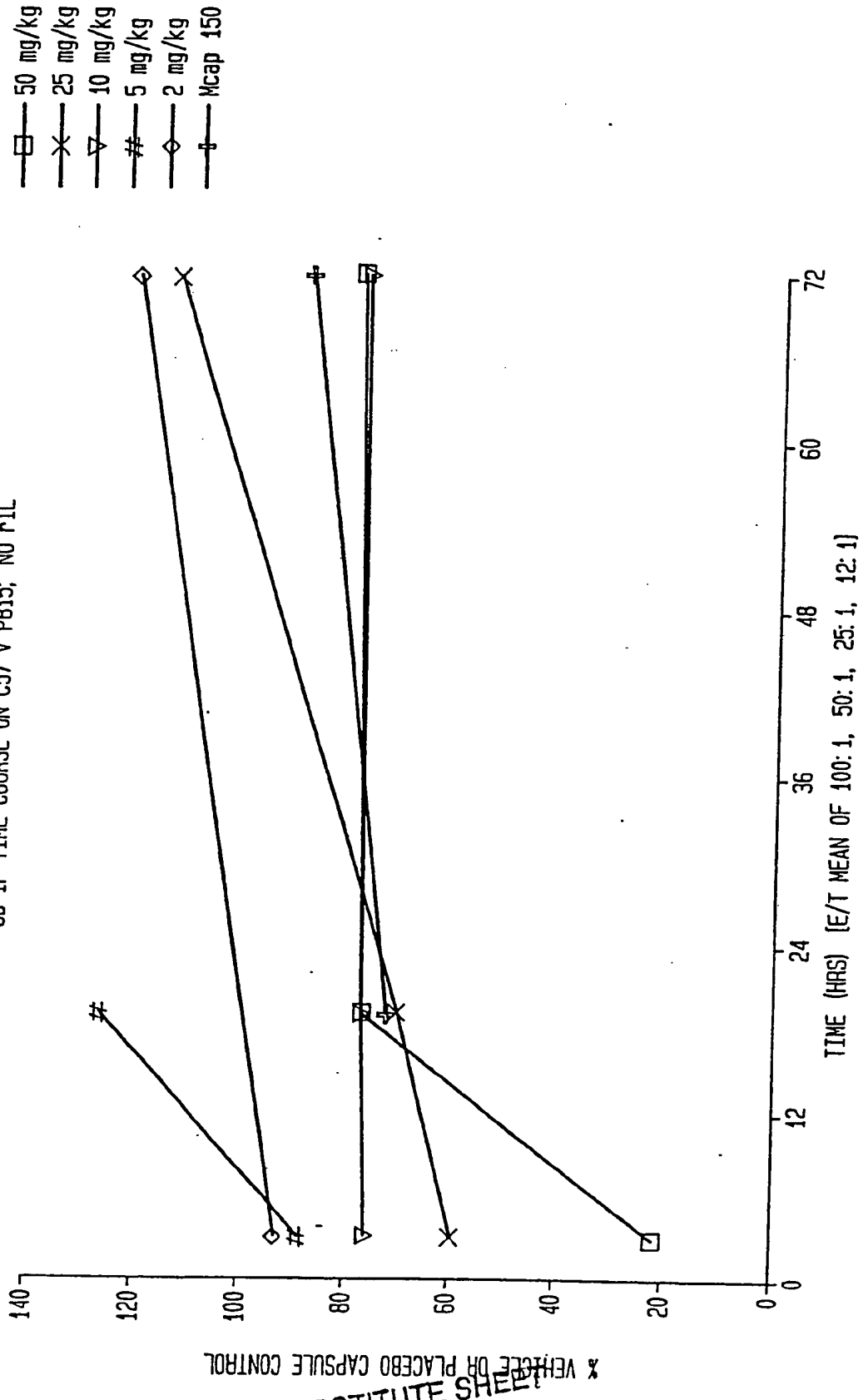


FIG. 10
CB IP TIME COURSE ON C57 v PB15; NO rIL

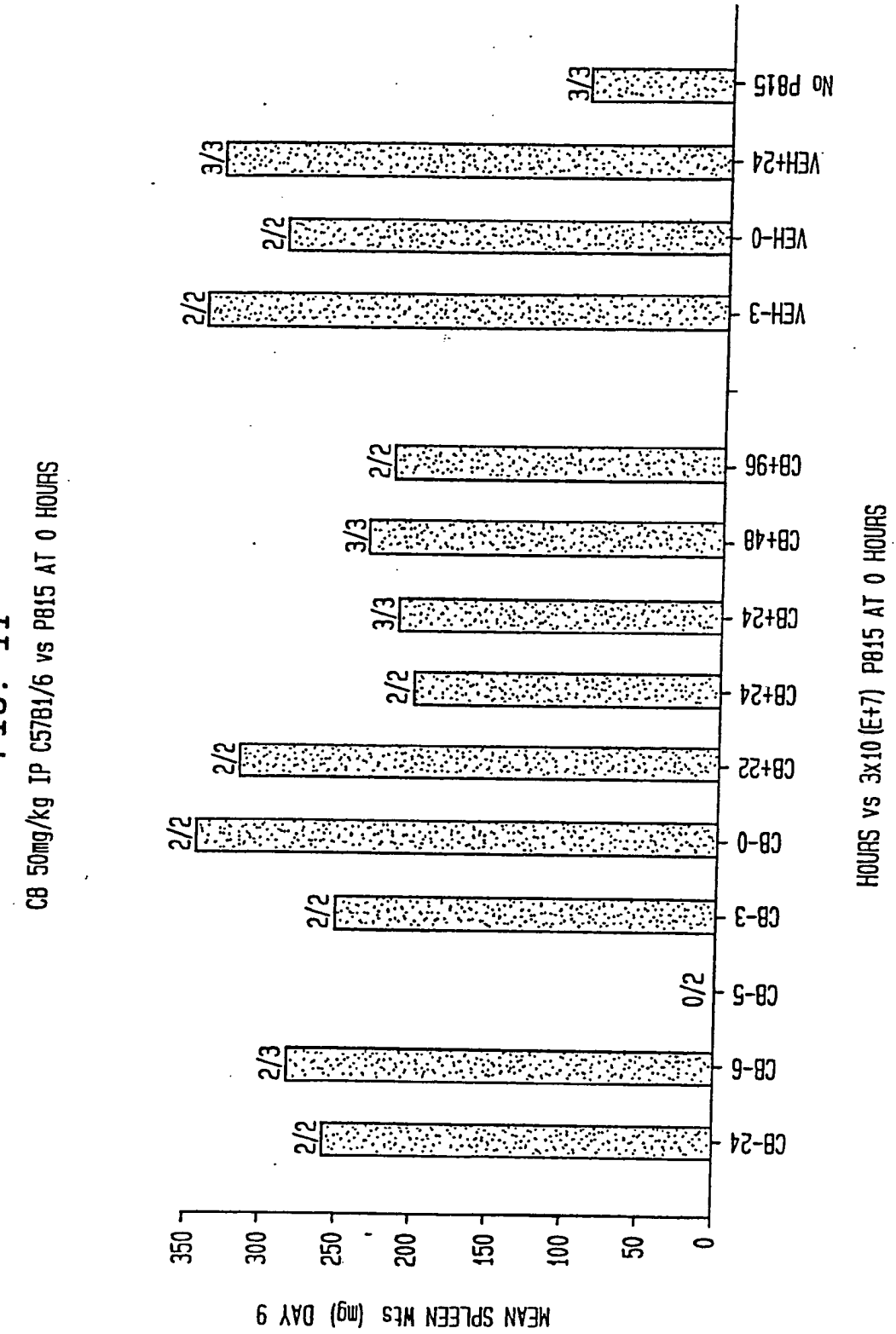


FIG. 11

CB 50mg/kg IP C57B1/6 vs P815 AT 0 HOURS

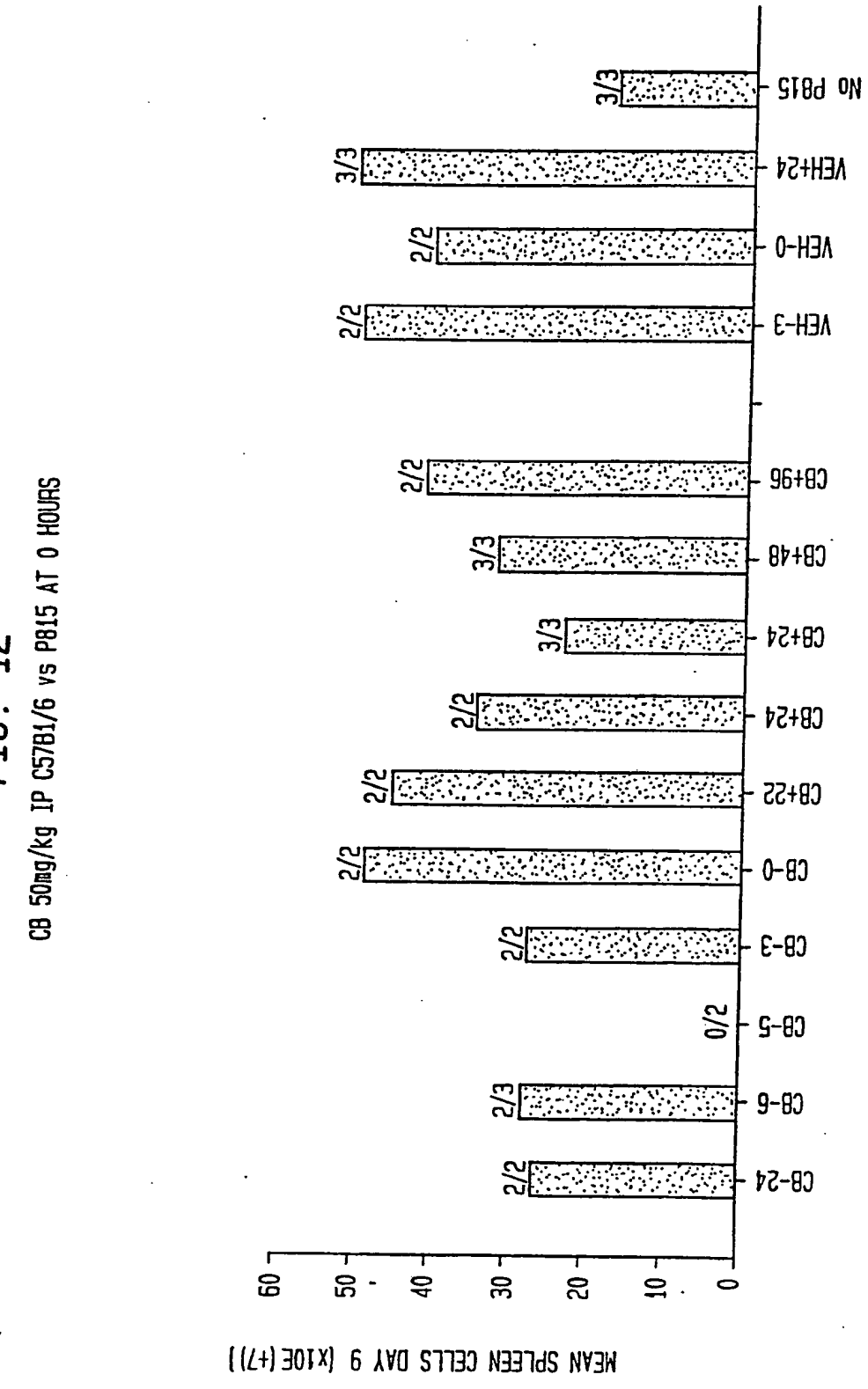


FIG. 12

CB 50mg/kg IP C57B1/6 vs P815 AT 0 HOURS

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02342

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/40
US CL.: 514/411

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	514/411

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

APS; CAS-on-line: Cytochalasin and hyperimmunity

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Chemical Abstracts, Volume 93, Number 25, issued 22 December 1980, E. Paunescu, "In vivo and in vitro comparative immunosuppressive effect on T cells of cytochalasin B(cyt.B) cyclophosphamide (CF), actinomycin D (Act.D) and two rifamycin (RF/SV) derivatives." See page 44, column 1, abstract number 230750U.	1 - 16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 July 1990	02 AUG 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Zahreh A. Fay

Form PCT/ISA/210 (second sheet) (May 1986)